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REMARKS

Amendments to the Specification:

The specification is amended to include reference to applications to which applicants previously claimed benefit of priority, but which were not earlier added in a formal amendment as a "Related Applications" paragraph. Applicants claimed benefit of priority to Provisional Application Serial Nos. 60/260,948, filed January 10, 2001 and 60/262,196, filed January 17, 2001, in a response to a Notice to File Missing Parts mailed June 26, 2002. This claim was acknowledged in an updated filing receipt mailed from the U.S. PTO on July 18, 2002, confirmation number 9824. No new matter is added by this amendment, nor is any change made to the effective filing date.

Amendments to the Claims

Claims 11, 23, and 31 have been cancelled. Applicants reserve the right to file a continuation application or take other such action to preserve rights to the embodiments of the cancelled claims or other unclaimed embodiments disclosed in the instant application.

Minor typographical errors (e.g., "n" to "$_n$") are amended in Claims 1, 3, 14, and 15 and the correct numbering of Claim 15. Double square brackets in chemical formulas in Claims 3, 14, and 15 (e.g., [[n]] in Claim 3) indicate deletions from the original claims, and are not part of the chemical formula.

Claims 1 and 12 are amended to the phrase "R4 is an *in vivo* hydrolyzable group"; likewise, Claim 12 is amended to include the phrase "R6 is an *in vivo* hydrolyzable group". Support can be found at page 6, line 15.

Claims 6, 18, and 26 are amended to include the phrase ", wherein the cancer cells are characterized by accumulation of glycosphingolipids, or changes in cancer cell levels of glycosphingolipids". Support can be found at page 11, lines 1-16.

Claims 6-10, 18-22, and 26-29 have been amended to delete the phrase "a composition comprising the". Claim 30 has been amended to deleted the phrase "a composition a".

Claims 8, 20, and 28 are amended to include the phrase ", wherein the infection is characterized by binding of the microbe, the virus, or a toxin thereof to glycosphingolipids on the patient's cells". Support can be found at page 9, line 33 to page 10, line 10.

Claims 9, 10, 21, 22, 29, and 30 are amended to include the phrase ", wherein the tumor is characterized by accumulation of glycosphingolipids, or changes in tumor cell levels of glycosphingolipids". Support can be found at page 11, lines 1-16.

Claim 24 is amended to incorporate the limitation "wherein n is an integer from about 1 to about 19" found on page 8, line 12.

Claims 26-30 have been amended to include the limitations of Claim 24.

New claims have been added. Support for new Claims can be found at page 9, line 33 to page 10, line 10 (32, 33, and 34); and page 6, line 15 (35 and 36).

Rejections under 35 U.S.C. § 112

In paragraph 4a, the Examiner rejects Claims 8, 20 and 28, directed to a method of treating microbial or viral infections. This rejection has been overcome by the present amendments to the claims. The Examiner asserts that the claims as written are drawn to "any and all microbial or viral infections". One skilled in the art will know that diseases treatable by the instantly claimed invention involve some aspect of the glucosyl ceramide synthase pathway. In particular, one skilled in the art is able to determine appropriate infections for treatment by identifying microbes, viruses, or related toxins that depend at least in part on binding to a glycosphingolipid (GSL) of a cell to be infected (page 9, line 33 to page 10, line 10). In fact, many such microbes, viruses, and related toxins are known, some of which are identified in paragraph 55 of the subject application. Such infections can reasonably be treated in a subject by reducing GSL production, thereby affecting the ability of the microbes, viruses, or related toxins to bind to GSLs. Without intending to limit the scope of the invention in any way, Applicants have amended Claims 8, 20, and 28 to include the phrase ", wherein the infection is characterized by binding of the microbe, the virus, or a toxin thereof to glycosphingolipids on the patient's cells" to more particularly claim and distinctly point out this aspect of the invention. Further, new claims 32, 33, and 34, depending respectively from Claims 8, 20 and 28 have been

added to direct the public to particular infections, e.g., due to "E. Coli, influenza A, or a verotoxin-producing organism" which can be treated by the invention.

In paragraph 4b, the Examiner maintains enablement rejections of Claims 6, 9-11, 18, 21-23, 26, and 29-31, asserting that the specification is not enabled for treating all cancers and/or tumors. This rejection has been overcome by the present amendments to the claims. As above, one skilled in the art knows that diseases treatable by the instantly claimed invention specifically involves one or more aspects of the glucosyl ceramide synthase pathway. In particular, cancers or tumors treatable by the instantly claimed invention are those that depend on the glucosyl ceramide synthase pathway. For example, page 11, lines 1-16 indicate that tumors can be dependent on GSL synthesis for growth, and accumulation of ceramide in treated tumors also slows growth or kills them, while GSL depletion can block the metastasis of tumor cells. Further, the secretion of GSL by tumors can adversely affect a patient's immune system which can negatively affect the patient's health, especially the immune system's response to the cancer or tumor cells. Also, tumor angiogenesis is correlated with high GSL levels. Without intending to limit the scope of the invention in any way, Applicants have amended Claims 6, 9, 10, 18, 21, 22, 26, 29, and 30 (Claims 11, 23, and 31 being cancelled), to include either the phrase ", wherein the cancer cells are characterized by accumulation of glycosphingolipids, or changes in cancer cell levels of glycosphingolipids" (Claims 6, 18, and 26) or ", wherein the tumor is characterized by accumulation of glycosphingolipids, or changes in tumor cell levels of glycosphingolipids" (Claims 9, 10, 21, 22, 29, and 30) to more particularly claim and distinctly point out this aspect of the invention.

In paragraph 4c, the Examiner maintains enablement rejections of Claims 9, 21, and 29, asserting that the specification is not enabled for treating multi-drug resistant cancers "because the Applicants do not indicate in the disclosure that the instant claimed compounds are superior to all other anti-cancer prodrugs". This requirement does not conform to the patent statutes. 35 U.S.C. §112, paragraph 1 requires enablement, written description, and disclosure of best mode; and 35 U.S.C. §101 requires that the claimed invention have utility. Superiority over the prior art is not a statutory requirement. On page 11, line 17 to page 12, line 9, an extended discussion is presented showing that compounds of the present invention have utility in blocking the growth

of multi-drug resistant tumors even at much lower dosages, in particular for tumors characterized by glycosphingolipid synthesis. Without intending to limit the scope of the invention in any way, Applicants have amended Claims 9, 21, and 29 as above to more particularly claim and distinctly point out this aspect of the invention.

In paragraph 4d, the Examiner maintains rejections of Claims 11, 23, and 31. Applicants respectfully request withdrawal of these rejections as these claims have been cancelled.

In paragraph 5b, the Examiner asserts that the phrase "R4 is a group that is selectively hydrolyzed in a target cell" in Claims 1 and 12 is unclear due to the incorporation of functional language. Without intending to limit the scope of the invention in any way, but merely to clarify, Applicants have amended Claims 1 and 12 to include the phrase "R4 is an *in vivo* hydrolyzable group" and likewise, Claim 12 to include the phrase "R6 is an *in vivo* hydrolyzable group", (e.g., as recited on page 6, line 15) to more particularly claim and distinctly point out this aspect of the invention. Further, claims 3 and 14, depending respectively from Claims 1 and 12, describe particular hydrolyzable groups recited on page 6, lines 14-27. Also, new Claims 35 and 36, depending respectively from Claims 3 and 14 have been added to direct the public to particular hydrolyzable groups wherein "n is 1". Further, one skilled in the art knows of many other *in vivo* hydrolyzable groups. For example, it is well known that ester groups are cleaved by esterases to expose a hydroxy group, e.g., the hydroxy group that would be exposed by cleaving R4 or R6 in the structure in Claim 12. Phenolic carbonates and carbamates are also degraded by cellular enzymes to yield phenols; see, for example, Exhibits A-I, e.g: Dittert, L. et al., J. Pharmaceutical Sci., 57:780 (1968) (Exhibit A); Dittert, L. et al., J. Pharmaceutical Sci., 57:828 (1968) (Exhibit B); Dittert, L. et al., J. Pharmaceutical Sci., 58:557 (1969) (Exhibit C); King, S. et al., Biochemistry, 26:2294 (1987) (Exhibit D); Lindberg, C. et al., Drug Metabolism and Disposition, 17:311 (1989) (Exhibit E); and Tunek, A. et al., Biochemical Pharmacology, 37:3867 (1988) (Exhibit F). In addition a variety of carbonate and carbamate groups are known which undergo spontaneous cleavage in solution at kinetically favorable rates; see, for example, Saari, W. et al., J. Medicinal Chem.,33:97 (1990) (Exhibit G); and Rattie, E. et al., J. Pharmaceutical Sci., 59:1741 (1970) (Exhibit H). Thus, the language "*in vivo* hydrolyzable

group" is clear and understandable to one of ordinary skill in the art because there are many such groups already known.

In paragraph 5c, the Examiner rejects Claims 3, 14, and 15 due to the phrase "...wherein n is at least 1..." as being indefinite for failing to set an upper limit. Different elements of a claim can be defined with greater or lesser particularity depending on the relation of each element to the novelty of the invention. For example, compound claims often recite a generic structure followed by a phrase such as "or pharmaceutically acceptable salts thereof". In such cases, the novelty is understood by one skilled in the art to be in aspects of the compound itself, and not in the peripheral pharmaceutically acceptable salts, which one skilled in the art will construe broadly to include every such salt known to the art. In the present case, the novel aspect of the parent claims of each of Claims 3, 14, and 15 is the "*in vivo* hydrolyzable group" of R^4 (Claim 1) or each of R^4 and R^6 (Claim 12). One skilled in the art will construe the group broadly to mean any such group known to the art, for example, those *in vivo* hydrolyzable groups described in the preceding paragraph. Further, Applicants note that one skilled in the art will certainly envision an upper practical limit on the size of such groups simply for reasons of physiological solubility and synthetic practicality; it is hard to imagine a case where one skilled in the art would recognize n of, e.g., 100 or 200 as anything but fanciful. Thus, the range is not unlimited to one skilled in the art. Also, a reasonable upper range based on solubility and synthetic practicality will be in the range of typical biological fatty acids, e.g., in one embodiment of another such group, a reasonable upper limit can be about 19 as described on page 8, lines 10-12. Further, new Claims 35 and 36, depending respectively from Claims 3 and 14 have been added to direct the public to particular hydrolyzable groups recited on page 6, lines 14-27, wherein support for "n is 1" is provided by the phrase "n is at least 1".

In paragraph 5d, the Examiner rejects Claim 24 as being indefinite for failing to recite the limits of n in the structural formulas therein. Applicants have amended Claim 24 to incorporate the limitation "wherein n is an integer from about 1 to about 19" found on page 8, line 12.

In paragraph 5e, the Examiner maintains rejections of Claims 8, 20, and 28, directed to treatment of infections; of 9, 21, and 29, directed to treating a drug resistant tumor; and of 10, 22, and 30, directed to treatment to reduce tumor angiogenesis. As noted above, one skilled in the art will know that diseases treatable by the instantly claimed invention involves some aspect of the glucosyl ceramide synthase pathway, in particular, as described above in the arguments and amendments for paragraphs 4a, 4b, and 4c. Applicants have presented arguments showing that in each case the conditions that can be treated are those that can be modified by administering the claimed glucosyl ceramide synthase inhibitor. One skilled in the art will know how to determine which conditions can be expected to be treatable by referring to the references cited in the specification. Further, dependent claims have been added indicating particular conditions that can be treatable. Thus, the rejection is believed to be overcome and its withdrawal is respectfully requested.

In paragraph 5h, the Examiner maintains rejections of Claims 11, 23, and 31. Applicants respectfully request withdrawal of these rejections as these claims have been cancelled.

In paragraph 6, Claims 2, 4, 5, 7, 13, 16, 17, 19, and 27 stand rejected as being dependent on rejected base claims. Upon withdrawal of rejections of the base claims, Applicants respectfully request withdrawal of rejections of the dependent claims.

Rejections under obviousness-type double patenting

In paragraph 8, the Examiner rejected Claims 12, 13, 24, and 25 under the judicially created doctrine of obviousness-type double patenting over claims 1, 2, and 4 of U.S. Patent No. 6,030,995 (the '995 patent). The Examiner indicated that this issue would be addressed when the claims were in condition for allowance. Applicants respectfully submit that Claims 12 and 13 are nonobvious because the hydrolyzable groups represented by R4 and R6 are not taught or suggested in the claims nor anywhere else in the '995 patent. Further, Claims 24 and 25 are novel over the '995 patent because the phenyl ring having the $-(CH_2)_nCH_3$ group (wherein n is an integer from about 1 to about 19) is not taught or suggested in the claims of the '995 patent, nor anywhere else in the patent. For example, Claim 1 of the '995 patent recites a group R1

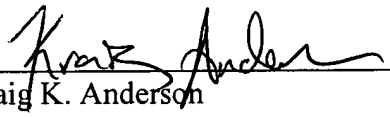
which is "an aromatic structure"; Claim 2 defines R_1 as a phenyl (unsubstituted); and Claim 4 of the '995 patent does not describe R_1. Thus, the rejection over the '995 patent is believed to be overcome and withdrawal of the obviousness-type double patenting rejection is respectfully requested.

CONCLUSION

In view of the above amendments and remarks, it is believed that all claims are in condition for allowance, and it is respectfully requested that the application be passed to issue. If the Examiner feels that a telephone conference would expedite prosecution of this case, the Examiner is invited to call the undersigned.

Respectfully submitted,

HAMILTON, BROOK, SMITH & REYNOLDS, P.C.

By 

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Dated: Apr. 19, 2004

activity studies in mice and rats suggested that the lipid and water solubilities of the compounds, rather than their enzyme-catalyzed hydrolysis rates, probably control the availability of acetaminophen following oral administration. These experiments provide additional evidence that the carbonate linkage may be an importantly useful one for the creation of variant physical and chemical properties in an entity with a singular pharmacologic action (11). This might then offer the opportunity to the formulator to choose among a variety of compounds with a singular pharmacologic action for the one which is: (*a*) amenable to physical formulation in a given dosage form; (*b*) stable in a given dosage form where all forms of the drug may not be equally stable; (*c*) satisfactory from the standpoint of taste or consumer acceptability; and (*d*) appropriate therapeutically from the standpoint of its time-action profile.

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Keyphrases

Acetaminophen prodrugs
Carbonate esters, acetaminophen–synthesis
Carboxylic acid esters, acetaminophen–synthesis
Hydrolysis rates, *in vitro*–acetaminophen prodrugs
Toxicity, oral–acetaminophen prodrugs
Analgesic activity–acetaminophen prodrugs

Acetaminophen Prodrugs II

Effect of Structure and Enzyme Source on Enzymatic and Nonenzymatic Hydrolysis of Carbonate Esters

By L. W. DITTERT*, G. M. IRWIN, C. W. CHONG, and J. V. SWINTOSKY*

Hydrolysis rates are reported for acetaminophen prodrugs with the structure CH_3CONH-ϕ-OCOOR at pH 7.4 in phosphate buffer alone or containing 1% human plasma or serum from several animal species. The hydrolysis rates in buffer decreased as the electrophilic character of the R group decreased. Dilute plasma or serum accelerated the hydrolysis; and the number of carbon atoms, the degree of chain branching, aromaticity, and chlorine substitution in the R group variously affected the degree of acceleration. In general, the sera of small rodents (mouse, guinea pig, and rat) were more potent catalysts of the hydrolyses of all types of acetaminophen carbonates than that of other animals (cat, dog, sheep, and rabbit) or human plasma.

THE METHODS of preparation and the physical properties of a series of carbonate esters of acetaminophen have been previously reported (1). These compounds were found to hydrolyze at various rates in dilute (2% v/v) human plasma solutions, and some of them had analgesic activity on the order of acetaminophen in rats. It was postulated that the analgesic activity was due to free acetaminophen released in the blood streams of the rats following oral administration of the prodrugs.

This report discusses the influence of the structure of acetaminophen carbonate prodrugs on the nonenzymatic hydrolysis of the compounds at pH 7.4 and on the enzymatic hydrolysis of the compounds catalyzed by blood plasma from humans and blood sera from species.

EXPERIMENT

Frozen citrated human blood was obtained in approximately from single donors through the Exchange. Frozen pooled ani tained from Colorado Serum Colorado.

Half-lives for the hydrolysis of carbonates at 37° in pH 7.4 (0.1 *M*), with and without huma serum, were determined by direc thermostatted cell compartment spectrophotometer. Fifty milli serum solution was warmed to 37 stoppered conical flask and a p spectrophotometer reference cell. of 95% ethanol containing an ap the acetaminophen carbonate w of a hypodermic syringe, and t gently until mixing was comp this mixture was transferred t the spectrophotometer and the until no further change was ob in which the reactions were ver sorbance values were calculat were determined from plots o *versus* time (see Fig. 1). In m trophotometer was set at 240 maximum of the esters), wher sorbance reflected the disappe In several experiments, the sp set at 300 mμ (the absorbanc aminophen), where an increas flected the appearance of ace given ester in a given enzyme s lives were obtained at the two

RESULTS AND DI

Half-lives for the hydrolysis of acetaminophen in pH 7.4 p

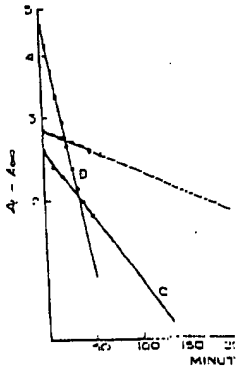

Fig. 1—Representative plots nature of the hydrolysis of carbo phen in pH 7.4 phosphate bu 1% (v/v) human plasma at determined at 240 mμ, the abs esters. (A∞ = absorbance at ti when the reaction is apparent lives are as follows—A, meth min.; B, butylcarbonate, t½

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Keyphrases

:n prodrugs
ers, acetaminophen–synthesis
cid esters, acetaminophen–

:es, *in vitro*–acetaminophen

–acetaminophen prodrugs
ity–acetaminophen prodrugs

s II

ı Enzymatic and

ıate Esters

ıd J. V. SWINTOSKY*

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humans and blood sera from various animal species.

EXPERIMENTAL

Frozen citrated human blood plasma (Type O+) was obtained in approximately 100-ml. quantities from single donors through the Philadelphia Serum Exchange. Frozen pooled animal sera were obtained from Colorado Serum Company, Denver, Colorado.

Half-lives for the hydrolysis of the acetaminophen carbonates at 37° in pH 7.4 phosphate buffer (0.1 *M*), with and without human plasma or animal serum, were determined by direct UV analysis in the thermostatted cell compartment of a Cary model 15 spectrophotometer. Fifty milliliters of the plasma or serum solution was warmed to 37° in a 125-ml. glass-stoppered conical flask and a portion placed in the spectrophotometer reference cell. One-half milliliter of 95% ethanol containing an appropriate amount of the acetaminophen carbonate was injected by means of a hypodermic syringe, and the flask was swirled gently until mixing was complete. A portion of this mixture was transferred to the sample cell of the spectrophotometer and the absorbance followed until no further change was observable. In cases in which the reactions were very slow, the final absorbance values were calculated. The half-lives were determined from plots of log Δ absorbance *versus* time (see Fig. 1). In most cases, the spectrophotometer was set at 240 mμ (the absorbance maximum of the esters), where a decrease in absorbance reflected the disappearance of the ester. In several experiments, the spectrophotometer was set at 300 mμ (the absorbance maximum of acetaminophen), where an increase in absorbance reflected the appearance of acetaminophen. For a given ester in a given enzyme system, the same half-lives were obtained at the two wavelengths.

RESULTS AND DISCUSSION

Half-lives for the hydrolysis of 11 carbonate esters of acetaminophen in pH 7.4 phosphate buffer (0.1 *M*), with and without 1% (v/v) human plasma, are shown in Table I. The rates of nonenzymatic cleavage of acetaminophen carbonate esters of straight-chain alcohols decreased with increasing chain length to four carbons; thereafter, they remained essentially constant at a minimum value.

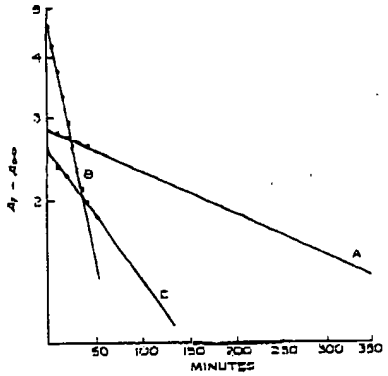
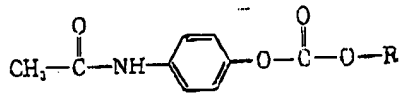


Fig. 1—Representative plots showing the first-order nature of the hydrolysis of carbonate esters of acetaminophen in pH 7.4 phosphate buffer (0.1 M) containing 1% (v/v) human plasma at 37°. Absorbances were determined at 240 mμ, the absorbance maximum of the esters. (A_t = *absorbance at time* t; A_∞ = *absorbance when the reaction is apparently complete.*) The *half-lives are as follows*—A, *methylcarbonate*, $t_{1/2}$ = *345 min.*; B, *butylcarbonate*, $t_{1/2}$ = *30 min.*; C, *isopropylcarbonate*, $t_{1/2}$ = *111 min.*

Table I—Half-lives for Hydrolysis of Carbonate Esters of Acetaminophen in pH 7.4 Phosphate Buffer (0.1 *M*)[a]



R	Half-lives, min. 1% Human Plasma	Half-lives, min. Buffer	$t_{1/2}$ buffer/ $t_{1/2}$ plasma
—CH_3	345	9,000	26
—C_2H_5	90	12,000	133
—C_4H_9	30	18,000	600
—C_6H_{13}	22	22,800	1036
—C_8H_{17}	28	19,800	707
—CH(CH₃)₂	111	18,000	162
—CH₂—CH(CH₃)₂	37	21,000	568
—C₆H₅	52	210	4.0
—C₆H₄—NH—C(=O)—CH₃	230	420	1.8
—CH_2—CH_2—Cl	60	1,320	26
—CH_2—C(—CH_3)—CH_3	52	210	4.0

[a] With and without 1% v/v human plasma (37°).

These results are in agreement with the findings of Gordon *et al.* (2), who studied the effect of the carbon chain length of aliphatic alcohols on the ammonolysis of their acetate esters. They found that the rate of ammonolysis decreased to a constant minimum value at alcohol carbon chain lengths of about five. These results suggest that the hydrolysis of the acetaminophen carbonates is subject to essentially the same electronic effects as the hydrolysis of carboxylic acid esters and of other carbonate esters (3); that is, the alkyl groups tend to act as "electron pumps" depolarizing the carbonyl group and making it less susceptible to nucleophilic attack. As with carboxylic acid esters, this inductive effect levels off at alkyl chain lengths of about four or five carbons.

The rates of enzymatic cleavage of the acetaminophen carbonates of straight-chain alcohols in 1% human plasma increased with increasing chain length up to about six carbons. The figures shown in Table I in the column headed $t_{1/2}$ buffer/$t_{1/2}$ enzyme, which indicate the degree of enzyme catalysis relative to the nonenzymatic rate, follow the same pattern. Adams and Whittaker (4), in studies on human plasma cholinesterase, reported that the optimum chain length for either the acyl group or the alkyl group in a homologous series of carboxylic acid esters is four carbons. Holstee (5) found that esters of fatty acids with seven carbons in the acyl chain are more susceptible to chymotrypsin catalyzed hy-

TABLE II—HALF-LIVES FOR HYDROLYSIS OF CARBONATE ESTERS OF ACETAMINOPHEN IN pH 7.4 PHOSPHATE BUFFER (0.1 *M*)

R	Buffer	Half-lives, min. ($t_{1/2}$ buffer/$t_{1/2}$ enzyme)[b]									
		Mouse	Guinea Pig	Rat		Cat	Human Plasma	Rabbit	Dog	Sheep
$-CH_3$	9000	30 (700)	51 (176)	95	(96)	294 (31)	345 (26)	79 (114)	40 (226)	335 (27)
$-C_2H_5$	12000	16 (800)	35 (343)	101	(119)	86 (140)	90 (133)	115 (104)	156 (77)	551 (22)
$-C_3H_7$	18000	6.9 (2610)	3.7 (4865)	6.7	(2687)	18 (1000)	30 (600)	50 (360)	27 (667)	520 (35)
$-CH(CH_3)_2$	18000	40 (450)	20 (900)	108	(167)	118 (153)	111 (162)	238 (76)	...	...
$-CH_2-CH(CH_3)_2$	21000	7.5 (2800)	5.6 (3750)	7.8	(2658)	12 (1760)	37 (568)	143 (147)	55 (382)	318 (66)
$-C_6H_5$	240	0.17 (1412)	0.25 (960)	1.3	(185)	10 (24)	52 (4.6)	...	100 (2.4)	165 (1.5)
$-NHCOC_6H_4CH_3$	420	1.5 (280)	1.5 (280)	3.6	(117)	11 (38)	130 (3.2)	71 (5.9)	111 (3.8)	63 (6.7)
$-CH_2-CCl_3$	240	0.21 (1143)	0.93 (258)	1.1	(218)	10 (24)	52 (4.6)	18 (13)	100 (2.4)	50 (4.8)

[a] Containing 1% serum from animals or 1% human plasma (37°). [b] The numbers in parentheses are $t_{1/2}$ buffer/$t_{1/2}$ enzyme ratios which represent the degree of enzymatic catalysis.

drolysis than esters of other fatty acids. The enzymes in human plasma responsible for the hydrolysis of acetaminophen carbonate esters appear to behave in a similar way. However, since human plasma contains a mixture of esterolytic enzymes, at least part of the dependence of the enzymatic rates on structure may be attributed to variations in the contributions made by the several plasma enzymes to the overall hydrolysis rates of the esters.

Half-lives for the hydrolyses of two acetaminophen carbonate esters of branched chain aliphatic alcohols are also shown in Table I. Branching slows both the nonenzymatic and the enzymatic hydrolysis reactions slightly, but the effect is less pronounced in the isobutyl than in the isopropyl case. The slowing effect of branching is probably due to inductive electronic effects similar to those observed by Gordon *et al.* (2) in their studies of the ammonolysis of acetate esters of ethanol, butanol, isopropanol, and isobutanol. Their results show a direct parallel with the nonenzymatic cleavage data for the corresponding acetaminophen carbonate esters shown in Table I.

Chain branching apparently has comparatively little effect on the degree of enzymatic catalysis of the acetaminophen carbonates by human plasma as shown by the closeness of the $t_{1/2}$ buffer/$t_{1/2}$ enzyme values for the ethyl and isopropyl derivatives and for the butyl and isobutyl derivatives. Thus, it would appear that branching in the aliphatic alcohol chain has much less of an effect on the human plasma catalyzed hydrolysis reaction than does the total number of carbons in the chain. This is an important finding with regard to the selection of derivatives for the preparation of carbonate ester prodrugs. Derivatives of branched alcohols generally have lower melting points and higher aqueous and nonaqueous solubilities than those of straight-chain alcohols (1). Since branching seems to have little effect on the hydrolysis of the carbonates, branched derivatives with their greater aqueous solubilities might be more rapidly available for oral absorption but equally susceptible to enzymatic hydrolysis as the straight-chain derivatives.

Table I shows hydrolysis data for the phenyl- and the *p*-acetaminophenylcarbonates of acetaminophen. The results suggest that aromatic groups apparently affected both the electronic state and the affinity of human plasma enzymes for these molecules. The phenolic carbonates were much more rapidly hydrolyzed in pH 7.4 buffer than the hexylcarbonate, probably because of the electrophilic character of the aromatic rings which polarize the carbonyl group and make it more susceptible to nucleophilic attack. The phenolic carbonates, however, were much less susceptible to enzymatic cleavage than is the hexylcarbonate, possibly because the hexyl group has a greater affinity for the catalytic sites on the esterolytic enzymes of human plasma.

The influence of chlorine substitution on the enzymatic and nonenzymatic hydrolysis of the ethylcarbonate of acetaminophen is illustrated in Table I. These data show that increasing chlorine substitution beta to the carbonate linkage progressively increased the nonenzymatic hydrolysis rate. The effect was quite pronounced in going from the ethyl to the monochloroethyl derivative and was apparently due to the capacity of chlorine to act as an "electron sink" causing increased polarization of the carbonyl

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The results of the studies on the phenolic carbonates and the chlorine substituted ethylcarbonates of acetaminophen suggest that prodrug carbonates of phenols and of alcohols containing electrophilic substituents are relatively more susceptible to nonenzymatic cleavage but relatively less susceptible to enzymatic cleavage than prodrug carbonates of aliphatic alcohols. Thus, phenols and electrophilic alcohols may form carbonate ester prodrugs which are less stable pharmaceutically and less labile to enzymatic cleavage *in vivo* than those formed by aliphatic alcohols.

Table II shows half-lives for the hydrolysis of eight carbonate esters of acetaminophen in pH 7.4 phosphate buffer (0.1 *M*) containing 1% blood serum from seven animals or 1% human plasma. Values for $t_{1/2}$ buffer/$t_{1/2}$ enzyme for each of the enzyme systems are shown in parentheses. The results show that serum from all the animals contains esterases capable of catalyzing the hydrolysis of the acetaminophen carbonate esters, although in several instances the catalysis is very weak.

For almost every species, the aliphatic carbonate esters can be ranked in the following order of susceptibility to enzymatic attack: butyl $\cong$ isobutyl > isopropyl $\cong$ ethyl > methyl. In serum from small rodents (mouse, guinea pig, and rat) the phenyl- and 2,2,2-trichloroethylcarbonates were about equally susceptible to enzymatic attack and the *p*-acetaminophenylcarbonate was somewhat less susceptible. In serum from the other animals and in human plasma, the *p*-acetaminophenylcarbonate was somewhat more susceptible to enzymatic attack than the phenyl- and 2,2,2-trichloroethylcarbonates.

For almost every compound, the sera of the animals can be ranked in the following approximate order of catalytic potency: mouse > guinea pig > rat > cat > human > rabbit > dog > sheep, although in several cases guinea pig and mouse could be interchanged in this ranking.

Augustinsson (6) reported wide species differences in both the concentrations and the specificities of the esterases normally found in the sera of animals and man. Since serum contains several esterolytic enzymes, each with its own specificities for the substrates, at least part of the dependence of the enzymatic rates on structure in a given sample of serum may be attributed to variations in the contributions made by the plasma enzymes to the overall hydrolysis rates. The specificities and the concentrations of the enzymes in the serum vary from species to species, and are probably largely responsible for the various hydrolysis rates observed for a given substrate in the sera of various animals.

Thus, it is not surprising that it is difficult to make broad generalizations based on the data of Table II. However, the following conclusions can be drawn: (*a*) if an acetaminophen carbonate ester has a comparatively large $t_{1/2}$ buffer/$t_{1/2}$ enzyme ratio in the serum of one species, it will probably also have comparatively large $t_{1/2}$ buffer/$t_{1/2}$ enzyme ratios in the sera of all species, and (*b*) the sera of small rodents seem to possess either higher concentrations of esterases, or esterases which have a greater affinity for acetaminophen carbonate esters than the sera of the other animals or human plasma.

All the compounds hydrolyzed in 1% human plasma. It would be expected that all would release free acetaminophen in the blood of humans fairly rapidly if they indeed reached the blood intact following oral administration. In almost every case, the compounds were hydrolyzed more rapidly in the serum of common laboratory test animals (except rabbit and dog) than in human plasma, and it would be expected that free acetaminophen would be rapidly released following oral absorption in these animals. Thus, human plasma is a convenient test system for studying the hydrolysis of carbonate ester prodrugs because hydrolysis in this system suggests that hydrolysis would also occur in the blood of humans and laboratory test animals *in vivo*. Lack of hydrolysis in dilute human plasma, however, would not necessarily mean that hydrolysis in the tissues of laboratory test animals and humans is not possible.

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Keyphrases

Acetaminophen prodrugs
Carbonate ester, acetaminophen—hydrolysis
Hydrolysis—enzymatic, nonenzymatic
UV spectrophotometry—analysis

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Carbonate Ester Prodrugs of Salicylic Acid

Synthesis, Solubility Characteristics, *In Vitro* Enzymatic Hydrolysis Rates, and Blood Levels of Total Salicylate Following Oral Administration to Dogs

By L. W. DITTERT*, H. C. CALDWELL, T. ELLISON†, G. M. IRWIN, D. E. RIVARD, and J. V. SWINTOSKY*

The methods of synthesis, solubilities, and partition coefficients for the ethyl-, butyl-, hexyl-, and 2,2,2-trichloroethylcarbonate esters of salicylic acid are reported. The solubility and partitioning characteristics of the ethyl- and trichloroethylcarbonates were similar to those of aspirin, whereas the butyl- and hexylcarbonates were more lipid soluble than aspirin. The *in vitro* hydrolysis rates of the compounds were also determined. The hydrolysis of the carbonate esters of salicylic acid were only slightly accelerated by 2 percent human plasma, and aspirin hydrolysis was not accelerated at all by this enzyme system. In the pseudocholinesterase and α-chymotrypsin systems, the hydrolysis of the butyl- and hexylcarbonates were accelerated to a much greater degree than those of the ethylcarbonate and aspirin. The results suggest that carbonates with 4 or 6 carbon alkyl chains fit the active sites of these esterolytic enzymes better than a carbonate with a 2 carbon alkyl chain or an acetate ester. Aspirin and the butyl-, hexyl-, and trichloroethylcarbonates were administered orally to dogs and the plasma levels of total salicylate were followed for 8 hr. The resulting blood level curves were virtually superimposable; all peaked at about 2 hr. and fell off at about the same rate. These results suggest that the prodrug carbonate esters are as readily absorbed as aspirin despite their different aqueous and lipid solubilities and that all the drugs including aspirin are converted to a common form, *i.e.*, free salicylate, within 2–3 hr. after oral administration.

FECAL BLOOD loss studies (1, 2) have shown that approximately 70% of persons taking aspirin experienced occult gastric bleeding averaging 5 ml. of blood per day, and Kelly (3) has reported that patients taking aspirin for long periods of time may develop severe iron deficiency anemia due to fecal blood loss. The mechanism by which aspirin causes gastric hemorrhage is a matter of considerable controversy (4). There is little doubt, however, that at least part of the problem is due to local irritation of gastric mucosa (5).

It was the purpose of our studies to seek prodrug derivatives of salicylic acid which would be nonirritant, would be readily absorbed, and which would be rapidly hydrolyzed in blood and other tissues to release free salicylate in the human blood stream. It was reasoned that carbonate esters of salicylic acid which are readily hydrolyzed by dilute human plasma, but which are less soluble in water and more soluble in lipids than aspirin, might be distributed and absorbed over a broader area of the gastrointestinal tract and might be less irritating to the gastric mucosa than aspirin.

This paper presents the synthesis, solubility, and partitioning properties, and *in vitro* hydrolysis rates of four carbonate ester prodrugs of salicylic acid. It also presents the blood levels of total salicylate following oral administration to dogs of three of the carbonate esters compared with aspirin.

EXPERIMENTAL

Chemical Synthesis—Melting points were determined in capillary tubes on a Thomas-Hoover apparatus and are uncorrected. Reported yields are of pure compound.

The following general procedure for synthesizing the compounds was adapted from the method of preparation of the methylcarbonate reported by Fischer (6). To an ice-cooled mixture of 85 Gm. (0.61 mole) of salicylic acid and 156 ml. (1.22 moles) of dimethylaniline in 500 ml. of dry benzene, the appropriate chloroformate (0.61 mole) was added over a period of 15 min. The ice bath was removed, and the mixture was stirred at room temperature for 2 hr. and washed with six 100-ml. portions of 10% HCl and three 100-ml. portions of water. The benzene solution was dried over anhydrous magnesium sulfate, and the solvent was removed. The remaining material was purified by crystallization from the appropriate solvent system (see Table II) after treatment with activated charcoal.

Particle Size Reduction—The purified compounds were micronized by one or more passes through a fluid energy mill (Tro: Products, Inc., Helmetta, N. J.) produced particles in the 2–20 μ s

Solubilities—The saturation compounds at 37° were determi and spectral grade cyclohexane (E bottles utilizing an assembly t scribed previously (7). HCl (0. assure that the compounds were ized and to reduce the degree of compounds during equilibratio samples were rotated for 6 hr., ar samples were rotated for 16 hr. (ov of the clear supernatant saturat withdrawn into hypodermic syrin pore filters held in Swinney filte pore Filter Corp.). The sampl spectrophotometrically.

Partition Coefficients—The pa of the compounds between cycloi HCl at 25° were determined usi apparatus previously described (HCl was used as the aqueous pha ments, the partition coefficients al

TABLE I—DOSING SCH

Compound	Mol. Wt.	Dose, mg./Kg.
Aspirin	180.16	75
Trichloroethyl[b]	313.54	120
Butyl[b]	238.24	90[c]
Hexyl[b]	266.80	111

[a] Dog weights: A = 12.8 Kg.; B Kg.; D = 12.0 Kg. [b] Carbonate of dose of the butyl carbonate is 10% less

TABLE II—CARBONATE ESTERS

R	Formula	Mol.
Ethyl (C_2H_5—)	$C_{9}H_{10}O_5$	210.
n-Butyl (C_4H_9—)	$C_{11}H_{14}O_5$	238.
n-Hexyl (C_6H_{13}—)	$C_{13}H_{18}O_5$	266.
Trichloroethyl (Cl_3C—CH_2—)	$C_{9}H_7Cl_3O_5$	313.

[a] Literature melting point is 95° (11)

TABLE III—ASPIRIN AND CARBON

Derivative	Solubilities in mg./ml., 37°: 0.1 N HCl[a]	Cyclohexane
Ethyl	0.7	1.1
n-Butyl	2.8	2.7
n-Hexyl	0.28	5.6
Trichloroethyl	1.1	0.24
Aspirin	6.3	0.06

[a] 0.1 N HCl was used in these experi and to reduce the degree of hydrolysis in (pH 7.4)/$t_{1/2}$ enzyme ratios which repre

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eduction—The purified com- nized by one or more passes through a fluid energy mill (Trost Jet Mill, Helme Products, Inc., Helmetta, N. J.). This procedure produced particles in the 2–20 μ size range.

Solubilities—The saturation solubilities of the compounds at 37° were determined in 0.1 *N* HCl and spectral grade cyclohexane (Fischer) in rotating bottles utilizing an assembly that has been described previously (7). HCl (0.1 *N*) was used to assure that the compounds were completely unionized and to reduce the degree of hydrolysis of the compounds during equilibration. The aqueous samples were rotated for 6 hr., and the cyclohexane samples were rotated for 16 hr. (overnight). Samples of the clear supernatant saturated solutions were withdrawn into hypodermic syringes through Millipore filters held in Swinney filter adapters (Millipore Filter Corp.). The samples were analyzed spectrophotometrically.

Partition Coefficients—The partition coefficients of the compounds between cyclohexane and 0.1 *N* HCl at 25° were determined using a method and apparatus previously described (8). Since 0.1 *N* HCl was used as the aqueous phase in these experiments, the partition coefficients shown in Table III are "true partition coefficients" of the unionized compounds.

In Vitro Hydrolysis Rates—Half-lives for hydrolysis of the compounds at 37° in 0.1 *M* phosphate buffer with and without enzymes were determined by a spectrophotometric method previously described (9). Frozen human blood plasma (Type O [b]) was obtained in approximately 100-ml. quantities from single donors through the Philadelphia Serum Exchange. Purified human pseudocholinesterase was obtained from Sigma Chemical Co., and α-chymotrypsin (salt free from EtOH) was obtained from Nutritional Biochemicals Co.

Blood Levels of Total Salicylate in Dogs—Purebred beagle dogs were used in a 4 × 4-crossover study with aspirin and the butyl- and trichloroethylcarbonates of salicylic acid. Each dog received an equimolar dose of drug per Kg. body weight according to Table I.

The drugs were administered *via* a stomach tube in single doses of finely ground powder suspended in 20 ml. of 0.5% tragacanth. Blood specimens were collected at 0, 15, 30, 60, 120, 240, 360, and 480 min. following drug administration, and total salicylate in the plasma was determined by the method of Cosmides, Stemler, and Miya (10).

TABLE I—DOSING SCHEDULE

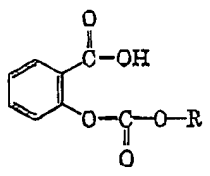
Compound	Mol. Wt.	Dose, mg./Kg.	Dosing Schedule[a] Day 1	4	8	11
Aspirin	180.16	75	A	B	C	D
Trichloroethyl[b]	313.54	120	B	C	D	A
Butyl[b]	238.24	90[c]	C	D	A	B
Hexyl[b]	266.30	111	D	A	B	C

[a] Dog weights: A = 12.8 Kg.; B = 8.6 Kg.; C = 8.1 Kg.; D = 12.0 Kg. [b] Carbonate of salicylic acid. [c] The dose of the butyl carbonate is 10% less than equimolar.

RESULTS AND DISCUSSION

The molecular weight, yields, melting points, elemental analyses, and recrystallization solvents for the four carbonate ester prodrugs of salicylic acid used in this study are shown in Table II. The ethyl derivative has been reported previously (11) but the others are new compounds. All are white

TABLE II—CARBONATE ESTERS OF SALICYLIC ACID: ELEMENTAL ANALYSES AND PHYSICAL PROPERTIES



R	Formula	Mol. Wt.	Yield, %	M.p., °C.	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found	Recrystallization Solvent
Ethyl (C_2H_5—)	$C_{10}H_{10}O_5$	210.190	40	93.5–94.5[a]	57.14	57.21	4.80	4.77	Chloroform-hexane
n-Butyl (C_4H_9—)	$C_{12}H_{14}O_5$	238.244	36	79.5–80.5	60.50	60.36	5.92	5.85	Carbon tetrachloride
n-Hexyl (C_6H_{13}—)	$C_{14}H_{18}O_5$	266.298	54	79.5–80.5	63.15	63.20	6.81	6.73	Carbon tetrachloride
Trichloroethyl (Cl_3C—CH_2—)	$C_{10}H_7Cl_3O_5$	313.527	42	126–128	38.31	38.37	2.25	2.29	Isopropanol-hexane

[a] Literature melting point is 95° (11).

TABLE III—ASPIRIN AND CARBONATE ESTERS OF SALICYLIC ACID: SOLUBILITIES, PARTITION COEFFICIENTS, AND HYDROLYSIS RATES

Derivative	Solubilities in mg./ml., 37° 0.1 *N* HCl[a]	Solubilities in mg./ml., 37° Cyclohexane	Cyclohexane/0.1 *N* HCl Partition Coefficient, 25°[a]	Half-Lives for Hydrolysis, 37° Phosphate Buffer (0.1 *M*) pH 7.4, hr.	Phosphate Buffer (0.1 *M*) pH 12, min.	Enzyme in Phosphate Buffer (pH 7.4, 0.1 *M*)[b] 2% Human Plasma, hr.	0.05% Human Pseudocholinesterase, hr.	0.05% α-Chymotrypsin, hr.
Ethyl	6.7	1.1	0.17	41	3.8	13.3 (3.1)	7.4 (5.5)	53 (—)
n-Butyl	2.8	2.7	1.0	29	4.5	11.7 (2.6)	1.23 (24)	1.38 (21)
n-Hexyl	0.28	5.6	20	15	4.8	8.2 (1.8)	1.33 (11)	0.25 (58)
Trichloroethyl	1.1	0.24	0.22	0.53	1.1	0.36 (1.5)		
Aspirin	5.3	0.06	0.06	10	1.5	21 (—)	3.3 (3)	6.3 (1.6)

[a] 0.1 *N* HCl was used in these experiments to assure that the compounds were completely unionized in the aqueous phases and to reduce the degree of hydrolysis in the aqueous phase during equilibration. [b] The numbers in parentheses are $t_{1/2}$ buffer (pH 7.4)/$t_{1/2}$ enzyme ratios which represent the degree of enzymatic catalysis.

crystalline, somewhat waxy, solids in their pure forms.

The solubilities of the carbonate esters and aspirin in 0.1 *N* HCl and in cyclohexane at 37° are shown in Table III along with the corresponding cyclohexane–0.1 *N* HCl partition coefficients at 25°. In these studies, 0.1 *N* HCl was used in place of water to assure that the compounds would not hydrolyze appreciably during equilibration and that they were completely unionized in the aqueous phases. Thus, the solubilities shown in Table III are those of the unionized compounds and the partition coefficients are "true partition coefficients."

As expected, the solubilities of the aliphatic carbonate esters decrease in 0.1 *N* HCl and increase in cyclohexane with increasing chain length. The measured partition coefficients show the expected increase with increasing chain length. The trichloroethyl derivative is less soluble in cyclohexane and much less soluble in water than the ethyl derivative. It might have been expected that the cyclohexane solubility of the trichloroethyl derivative would be greater than it is, since trichloroethyl groups tend to be more lipophilic than ordinary ethyl groups, but the trichloroethylcarbonate of salicylic acid has a higher melting point than the other derivatives indicating a stronger crystal lattice which tends to reduce solubility in all solvents.

Predictions of the relative availabilities of the compounds for oral absorption based on the solubilities and partition coefficients shown in Table III must take into account the fact that they probably do not have precisely equivalent pKa values; therefore, at pH's near their pKa's, some of the compounds might show relatively higher or lower apparent partition coefficients than would be expected on the basis of the true partition coefficients shown in Table III. Also, because the compounds are weak acids, the aqueous solubilities will increase with increasing pH. However, the fact that solubilities shown in Table III are of a reasonable order of magnitude suggests that if the drugs are finely ground and properly wetted by water, they should all be readily absorbed following oral administration.

If the solubilities and partition coefficient of aspirin are compared with those of the carbonates, it might be predicted that the ethyl derivative would have gastrointestinal absorption characteristics similar to those of aspirin. In subsequent tests (12) it was found that the ethyl derivative was almost as irritating to the gastric mucosa of rats as aspirin itself. It would seem that salicylate derivatives with relatively high aqueous solubilities and relatively low lipid solubilities are more irritating to the gastric mucosa than those which are less soluble in water and more soluble in lipids.

Table III also shows enzymatic and nonenzymatic hydrolysis data for the four carbonate esters and aspirin. The aliphatic carbonates are more stable toward hydrolysis in buffer than aspirin. The trichloroethylcarbonate is less stable, probably because the chlorine atoms act as good "electron sinks" drawing electrons away from the carbonyl carbon and making it susceptible to nucleophilic attack.

In 2% human plasma in pH 7.4 buffer, the aliphatic carbonates and aspirin are very slowly hydrolyzed. The values for $t_{1/2}$ buffer/$t_{1/2}$ enzyme, shown in parentheses in Table III, represent the degree of enzymatic catalysis and show that the esterases of human plasma have very little catalytic effect on the hydrolysis of these compounds. Even though the hydrolysis of the trichloroethylcarbonate derivative is relatively rapid, the degree of enzymatic catalysis is practically nil. In 0.05% "purified" human pseudocholinesterase and 0.05% α-chymotrypsin (from bovine pancreas) the ethylcarbonate derivative and aspirin are hydrolyzed relatively slowly and the degree of enzymatic catalysis in each case is relatively small. On the other hand, the butyl- and hexylcarbonates are hydrolyzed relatively rapidly by these enzymes and the degree of enzymatic catalysis is considerably larger. These prodrugs, however, are not nearly as susceptible to enzymatic hydrolysis by these enzymes as is 4-acetamidophenyl 2,2,2-trichloroethyl carbonate. For this carbonate ester prodrug of acetaminophen, the $t_{1/2}$ buffer/$t_{1/2}$ enzyme values were 1680 for 0.05% human pseudocholinesterase and 646 for 0.05% α-chymotrypsin (12). Of all the enzymes studied, these two were the most powerful catalysts for the hydrolysis of carbonate esters of acetaminophen, but they are apparently much weaker catalysts for the hydrolysis of carbonate esters of salicylic acid.

Augustinsson and Nachmansohn (13) reported the presence of an aspirin esterase in human blood which is clearly different from the pseudocholinesterase of serum. Morgan and Truitt (14) studied the *in vitro* hydrolysis of aspirin in serum from various species of laboratory animals and humans, and they reported that the aspirin esterase activity of human blood is very weak. The esterases of human blood, having a somewhat greater affinity for carbonate esters, than for aspirin, hasten the hydrolysis of these esters.

The *in vitro* enzyme hydrolysis studies suggested that free salicylate might be released at different rates following oral administration of aspirin and the carbonate prodrugs, but there are many esterolytic enzymes and many sites in the body other than the blood stream where hydrolysis of these compounds might occur. To investigate the release rates and sites of hydrolysis and to study the influence of the physical properties of the carbonate prodrugs on their *in vivo* performance, the prodrugs and aspirin were administered orally to dogs, and the blood levels of salicylate were followed. The analytical method employed (10) converts the carbonate esters and aspirin to free salicylate, therefore, the blood levels were obtained in terms of total salicylate.

The average total salicylate plasma levels in dogs following oral administration of aspirin and the butyl-, hexyl-, and trichloroethylcarbonates of salicylic acid are shown in Fig. 1. The curves in Fig. 1 show that the drugs are nearly identical with respect to the plasma salicylate levels they produce in dogs. All curves reach a peak near 20 mg. % at 2 hr. and then fall off at about the same rate. The minimum and maximum individual blood levels for all drugs overlap at all time points.

These results suggest that the solubility and partitioning properties of these prodrug carbonate esters did not grossly affect their gastrointestinal absorption rates. A possible exception is the hexyl derivative which appears to have a somewhat slower absorption rate than the others. However, the absorption rate of this compound is apparently fast

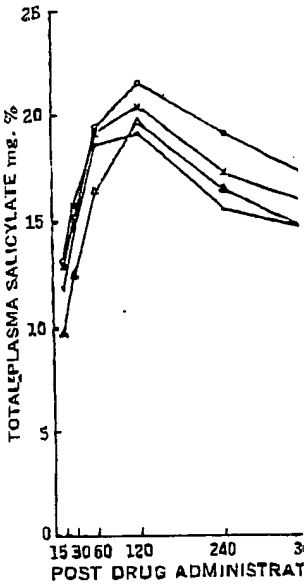


Fig. 1—Plot showing plasma levels in purebred beagle dogs following o of equimolar doses of aspirin (- trichloroethylcarbonate of salicylic mg./Kg.); butylcarbonate of salicyl mg./Kg.); and hexylcarbonate of sa 111 mg./Kg.). The study was ca lo a 4×4 crossover plan, and each average of four plasma levels. T maximum individual plasma levels drugs at all time poi

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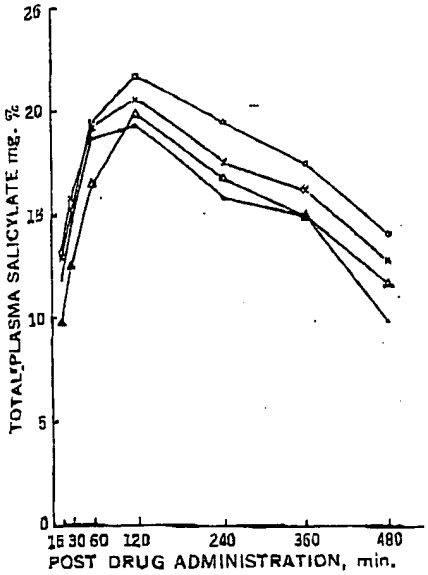

Fig. 1—Plot showing plasma levels of total salicylate in purebred beagle dogs following oral administration of equimolar doses of aspirin (.—., 75 mg./Kg.); trichloroethylcarbonate of salicylic acid (O—O, 130 mg./Kg.); butylcarbonate of salicylic acid (×—×, 90 mg./Kg.); and hexylcarbonate of salicylic acid (Δ—Δ, 111 mg./Kg.). The study was carried out according to a 4×4 crossover plan, and each point represents the average of four plasma levels. The minimum and maximum individual plasma levels overlapped for all drugs at all time points.

enough to catch up with the other carbonates and aspirin at the 2-hr. point. The fact that all the blood levels follow the same pattern and fall off at about the same rate suggests that beyond 2 or 3 hr., aspirin and the carbonate prodrugs of salicylic acid are all in the same form, that is, they have been completely converted to free salicylate. Thus, it would appear that aspirin and the three carbonates are rapidly converted to free salicylate *in vivo*, and it might be expected that the pharmacologic activities of the three carbonates would be very similar to those of aspirin.

Based on these findings, extensive pharmacologic evaluations of the butyl-, hexyl-, and trichloroethylcarbonates were undertaken. Future publications in this series will deal with their analgesic, antipyretic, and anti-inflammatory activities and their gastrointestinal irritation liabilities compared with aspirin.

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Keyphrases

Salicylic acid carbonate esters—prodrugs
Carbonate esters salicylic acid—synthesis
Blood levels—salicylate
Hydrolysis rates—*in vitro*
Solubility—salicylic acid carbonate esters

Hydrolysis of 4-Acetamidophenyl 2,2,2-Trichloroethyl Carbonate by Esterolytic Enzymes from Various Sources

LEWIS W. DITTERT, GEORGE M. IRWIN*, ELISABETH S. RATTIE, CLIFFORD W. CHONG, and JOSEPH V. SWINTOSKY

Abstract □ Hydrolysis rates were determined for ATC in pH 7.4 phosphate buffer containing human plasma from a number of individuals; human plasma treated and stored in various ways; several Cohn fractions of human plasma; human plasma treated with various esterase inhibitors; and a number of commercially available enzymes. The variation among individual plasma samples was observed, as well as the way in which blood type, plasma concentration, lyophilization, freezing, thawing, storing the plasma at 25°, ethanol concentration, and substrate concentration influenced the catalytic potency of human plasma. Studies with the Cohn fractions and esterase inhibitors suggested that pseudocholinesterase was primarily responsible for the enzymatic activity of human plasma with respect to ATC hydrolysis. Some proteolytic enzymes were also found to be potent catalysts of ATC hydrolysis. It was concluded that, following oral administration, ATC would be exposed to many enzymes that are potent catalysts for its hydrolysis, both in the gastrointestinal tract and following absorption and distribution in body tissue.

Keyphrases □ 4-Acetamidophenyl 2,2,2-trichloroethyl carbonate (ATC)—hydrolysis □ Enzymes, human plasma—ATC hydrolysis □ Pseudocholinesterase—ATC esterolytic hydrolysis

The authors' experience has indicated that acetaminophen and other drugs possessing the hydroxyl group may be converted to various carbonate esters and still retain the intrinsic therapeutic activity of the parent drug *in vivo*. Because of this, it is possible to prepare a series of compounds with approximately equivalent therapeutic activity on a molar basis, but with widely ranging differences in physical-chemical properties. These differences could be of importance to the pharmacist preparing variant forms of a drug because they might affect dissolution rate, physical form, and taste; they might also influence the absorption and stability of a drug, as well as the dosage form in which it can be employed.

The compound, 4-acetamidophenyl 2,2,2-trichloroethyl carbonate (ATC), is an interesting carbonate ester variant of acetaminophen. The hydrolysis rates of ATC in buffer solutions and in buffers containing plasma and intestinal fluid from rats and humans have been reported (1). It has also been established that the hydrolysis rates of carbonate and carboxylic acid esters of acetaminophen with a variety of structures are accelerated by the blood sera of humans and animals (2). Presumably, the catalysis produced by these body fluids is due to esterolytic enzymes; however, many proteolytic and other types of enzymes can also function as esterases. For this reason, it was of interest to determine what factors influence the *in vitro* catalytic potency of a crude enzyme system such as human plasma; and what relative catalytic potencies are obtained with various enzyme systems when ATC is the substrate.

Table I—Half-Lives for the Hydrolysis of ATC in 2% Human Plasma from Various Donors (0.1 *M*, pH 7.4 Phosphate Buffer, 37°C.)

Sample No.	Blood Type	Half-Life (min.) (Average ± Mean Deviation)
12446	AB+	10.7
		12.0
		11.8 (av. 11.5 ± 0.5)
12449	O+	13.0
		10.2 (av. 11.6 ± 1.4)
12450	A+	19.5
		21.3 (av. 20.4 ± 0.9)
12448	B+	20.0
		17.1
		17.8 (av. 18.3 ± 1.1)
GMI	A−	15.0
S-12593	O−	14.3
S-12589	O+	21.5
S-12590	O−	19.0
LWD	O+	17.7
S-12591	A−	19.3
S-12594	A−	20.3
S-12592	A+	16.0
69255	B+	21.3
P-69334	B+	15.8
69338	B+	21.0
P-69254	B+	31.3
P-69332	B+	20.6
S	B+	25.8
		Av. 17.6 ± 4 min. (limits 10.2–31.3)

EXPERIMENTAL

Frozen citrated human blood plasma of various blood types from individual donors, and lyophilized Cohn fractions of human plasma were obtained.[1] Lyophilized whole human plasma was prepared from 110 mL of Type O+ citrated human plasma. The other materials used were: human pseudocholinesterase,[2] horse pseudocholinesterase,[3] crystalline human serum albumin and the purified enzymes,[4] physostigmine sulfate and tetraethyl pyrophosphate (TEPP),[5] and sodium ethylenediaminetetraacetate (EDTA).[6]

Half-lives for the hydrolysis of ATC at 37° in 100% human plasma and in pH 7.4 phosphate buffer (0.1 *M*) containing 25, 50, and 75% human plasma or 7.5% lyophilized human plasma were determined by a chromatographic procedure previously described (1). All other half-lives were determined by a direct UV procedure previously described (1). All reactions followed apparent pseudo first-order kinetics.

RESULTS AND DISCUSSION

Half-lives for the hydrolysis of ATC in pH 7.4 phosphate buffer (0.1 *M*) containing 2% human plasma from 18 donors are shown in Table I. The half-lives varied from a minimum of 10.2 min. to a maximum of 31.3 min., with an average half-life of

[1] Through the Philadelphia Serum Exchange.
[2] Cutter Laboratories, Berkeley, Calif.
[3] Armour Pharmaceutical Co., Kankakee, Ill.
[4] Nutritional Biochemicals Corp., Cleveland, Ohio.
[5] K & K Laboratories, Jamaica, N. Y.
[6] Fisher Scientific Co., Fair Lawn, N. J.

Table II—Effect of Human Plasma Concentration on Hydrolysis Rate of ATC (0.1 *M*, pH 7.4 Phosphate Buffer, 37°C.)

Plasma Concn., %		Half-Life, min.
Liquid		
2		19.5
4		13.9
6		11.2
25		6.1
50		4.2
75		3.4
100		2.9
Lyophilized (Liquid Equivalent, %)		
0.05	(0.667)	28
0.1	(1.33)	35
0.2	(2.67)	17.2
0.3	(4.00)	11.9
0.5	(6.67)	12.0
7.5	(100)	7.5

about 18 ± 4 min. There was considerable variation among individual samples of plasma with respect to their ability to accelerate the hydrolysis of ATC, but there appeared to be no correlation between blood type and catalytic potency. The results shown in Table I also indicate that, in repeated experiments with the same sample of plasma, a mean deviation of 5 to 10% in the measured half-life can be expected with the experimental technique employed.

Table II shows the effect of plasma concentration on the half-life of ATC at pH 7.4. As the concentration of either liquid or lyophilized human plasma was increased, the rate of hydrolysis of ATC increased (half-life decreased), but the increase was not directly proportional to the increase in plasma concentration with either material. Thus, one can expect ATC to be very rapidly hydrolyzed to free acetaminophen in blood plasma *in vivo*, but one would not necessarily expect it to be hydrolyzed 50 times faster in blood plasma *in vivo* than it is in 2% plasma solution *in vitro*. The half-life of ATC in a given concentration of lyophilized plasma was roughly equivalent to the half-life in an equivalent concentration of liquid plasma; this showed that lyophilization did not destroy or inhibit to any great extent the enzymes of human plasma that catalyze the hydrolysis of ATC.

To study the hydrolysis of ATC in plasma and the various enzyme systems using a direct UV procedure, it was necessary to dissolve the drug initially in a water-miscible solvent, to use a concentration of ATC in the final enzyme mixture which would optimize the UV analysis, and to freeze and thaw the plasma samples, perhaps several times, before use. These factors were investigated to see if they had a profound influence on the half-life of ATC in 2% human plasma in pH 7.4 phosphate buffer.

Table III—Effect of Ethanol, Substrate Concentrations, and Various Conditions of Plasma Storage on Hydrolysis Rate of ATC in 2% Human Plasma Solutions (0.1 *M*, pH 7.4 Phosphate Buffer, 37°C.)

Effect of Ethanol Concn. (0.03 mg./ml. ATC)	
Ethanol Concn.	Half-Life, min.
0.75%	40
1	30
2	30
3	41
Effect of ATC Concn. (1% ethanol)	
ATC Concn., mg./ml.	Half-Life, min.
0.01	36
0.015	36
0.03	28
0.04	33.6
Effect of Conditions of Plasma Storage (1% ethanol, 0.03 mg./ml. ATC)	
History	Half-Life, min.
1. Freshly drawn plasma	23
2. No. 1, frozen overnight, thawed rapidly[a]	22.1
3. No. 1, frozen overnight, thawed slowly[b]	24
4. No. 3, stored 19 hr. at 25°	29
5. No. 3, stored 43 hr. at 25°	25

[a] A 20-ml. vial was placed in 37° water until thawed. [b] A 20-ml. vial was placed in air at 25° until thawed.

Table IV—Catalytic Potency of Various Cohn Fractions of Human Plasma on the Hydrolysis of ATC (0.1 *M*, pH 7.4 Phosphate Buffer, 37°C.)

Cohn Fraction	Concn., %	Half-Life, min.
I	0.1	—[a]
II	0.028	—[a]
III-0	0.027	56
III-1	0.035	—[a]
III-2	0.033	—[a]
III-3	0.033	—[a]
IV	0.1	10.6
IV-1	0.032	—[a]
IV-4	0.036	20.8
V	0.1	21.7
Crystalline human serum albumin	4	99

[a] Half-life over 240 min. No enzymatic activity.

The results are shown in Table III. Ethanol concentrations between 0.75 and 3% and ATC concentrations between 0.01 and 0.04 mg./ml. in the final enzyme mixture had no apparent effect on the hydrolysis rate; the half-lives were within experimental error. The half-lives produced by 2% solutions of human plasma that were freshly drawn, frozen and thawed rapidly, frozen and thawed slowly, or stored at 25° for 19 and 43 hr. were also within experimental error. These results suggest that low concentrations of ethanol and ATC had very little effect on the human plasma enzymes which hydrolyze ATC, and that storing plasma in small containers in a freezer and rapidly thawing it shortly before use can be expected to have little effect on the catalytic potency of this crude enzyme source with respect to hydrolysis of acetaminophen carbonate ester prodrugs.

Table IV shows the catalytic potency of several Cohn human plasma fractions (3) with respect to hydrolysis of ATC. The activity appears to be concentrated in the III-0, IV-4, and V fractions; the other fractions studied had no activity. Fraction V is composed mainly of albumin, which has been reported to be a catalyst in some esterolytic reactions (4). However, crystalline human albumin at relatively high concentrations (4%) showed practically no catalytic effect on the hydrolysis of ATC and it may be concluded that the esterolytic activity of Cohn fraction V shown in Table IV was not due to catalysis by albumin but by enzymes left in Fraction V by the Cohn fractionation procedure.

Cohn fractions III and IV are composed mainly of globulins and contain aromatic esterase (A-esterase) and pseudocholinesterase (C-esterase) which are reported to be the major esterolytic enzymes of human plasma (5). These enzymes are not separated in the Cohn fractionation procedure, however, and the experiments with the Cohn fractions were unable to distinguish which of these enzymes is responsible for the hydrolysis of ATC. Pseudocholinesterase has been shown to be identical to "procainesterase" (6), and it was of interest to know if this esterase is also involved in the hydrolysis of carbonate prodrug esters such as ATC. Pseudocholinesterases are inactivated by physostigmine and TEPP (7), but aromatic esterase is unaffected by these inhibitors. However, for aromatic esterase to exert its activity, calcium ions must be present in the medium (8). Table V

Table V—Effect of Calcium Ion and Cholinesterase Inhibitors on the Hydrolysis Rate of ATC in 2% Liquid Human Plasma (0.1 *M*, pH 7.4 Phosphate Buffer, 37°C.)

Concn. of Ca^{++} or Inhibitor	Half-Life, min.
None	19
10^{-4} *M* $CaCl_2$	18
10^{-4} *M* EDTA	17
10^{-4} *M* Physostigmine sulfate	—[a]
10^{-4} *M* TEPP	—[a]

[a] Half-life over 240 min. No enzymatic activity.

Table VI—Half-Lives for Hydrolysis of ATC in Dilute Solutions of Purified Human Pseudocholinesterase (0.1 *M*, pH 7.4 Phosphate Buffer, 37°C.)

Concn., %	Half-Life, min.
0.0025	9.2
0.005	5.8
0.01	1.1
0.02	0.63
0.03	0.32

shows that the esterolytic activity of 2% human plasma with respect to ATC hydrolysis was completely destroyed by $10^{-4}M$ physostigmine sulfate or $10^{-4}M$ TEPP, whereas the half-life for hydrolysis was unaffected by $10^{-4}M$ calcium chloride or $10^{-4}M$ EDTA. These results support the implication that human plasma pseudocholinesterase is primarily responsible for the esterolytic activity of human plasma with respect to ATC hydrolysis.

Human pseudocholinesterase is available commercially in a purified form, and Table VI shows half-lives for the hydrolysis of ATC in pH 7.4 phosphate buffer containing relatively low concentrations of this material. The results show that the reaction rate was roughly proportional to enzyme concentration in the 0.01 to 0.03% range. Half-lives of about 20 sec. were obtained for ATC in 0.03% solutions, confirming that human pseudocholinesterase is a very potent enzyme with respect to the hydrolysis of ATC. A comparison of the catalytic potency of human pseudocholinesterase with that of other commercially available enzymes (Table VII) shows that human and horse pseudocholinesterases were among the most potent catalysts, with human enzyme the most potent of all the enzymes studied.

Proteolytic enzymes, especially the chymotrypsins and trypsin, were also potent catalysts. As might be expected, pepsin and papain showed weak activity in this experiment; these enzymes are most active at more acidic pH's. It is also not surprising that acetylcholinesterase was only a very weak catalyst, since this enzyme is virtually specific for acetylcholine and has very little effect on most other esters (9).

The remaining enzymes listed in Table VII showed little or no activity under the conditions of this experiment. It would be difficult to explain why each enzyme behaved as it did, and explanations based on this brief study would have very little meaning. However, it can be concluded that, following oral administration, ATC will be exposed to many enzymes which are potent catalysts for its hydrolysis, particularly the proteolytic enzymes and pseudocholinesterase; it might also be surmised that other prodrug carbonate and carboxylate esters of acetaminophen, or other parent drugs which contain a hydroxyl group, may also be hydrolyzed by these enzymes.

Table VII—Half-Lives for Hydrolysis of ATC in Dilute Solutions of Various Purified Enzymes (0.05%) (0.1 *M*, pH 7.4, Phosphate Buffer, 37°C.)

Enzyme	Half-Life, min.	Rate Relative to Buffer Alone
Human plasma cholinesterase (0.03%) (Cutter Labs.)	0.3	800
α-Chymotrypsin	0.65	370
β-Chymotrypsin	0.56	430
γ-Chymotrypsin	0.51	470
Δ-Chymotrypsin	0.53	450
Horse pseudocholinesterase (Armour Co.)	1.4	171
Acylase (hog kidney)	2.2	109
Trypsin (1–300)	3.0	80
Pancreatin	15	16
Peptidase	20	12
Malt Diastase	24	10
Pectin esterase	27	9
Maltase	38	6
Proteinase	66	4
Acetyl cholinesterase (bovine erythrocyte)	74	3
Erepsin	78	3
Lysozyme	99	2.3
Papain	124	2
Pepsin (1–20,000)	185	1.3
Penicillinase	> 240 (no activity)	—
Pepsin (blood group A free)	> 240 (no activity)	—
Carbonic anhydrase	> 240 (no activity)	—
Enterokinase	> 240 (no activity)	—
Lipase	> 240 (no activity)	—
Hog kidney extract	> 240 (no activity)	—
No enzyme	> 240 (no activity)	—

among the commercially available enzymes studied at pH 7.4. Thus, enzymes found in the gastrointestinal tract, liver, and blood are capable of rapidly hydrolyzing ATC and would probably hydrolyze other carbonate and carboxylic acid prodrug esters of acetaminophen as well. Consequently, free acetaminophen should be rapidly released following oral administration and dissolution of these types of compounds. It might also be expected that the pseudocholinesterases and the chymotrypsins would be active catalysts of carbonate and carboxylic acid ester prodrugs of other parent drugs which contain the hydroxyl group.

SUMMARY

1. Individual specimens of human plasma varied in their catalytic potencies with respect to the hydrolysis of ATC, but there appeared to be no correlation between blood type and catalytic potency.

2. The hydrolysis rate of ATC in pH 7.4 phosphate buffer containing human plasma increased with increasing plasma concentration, but the increase was not proportional to the plasma concentration over the entire concentration range. Thus, the rate in 100% plasma was about seven times faster than the rate in 2% plasma.

3. Lyophilization of plasma, ethanol concentrations between 0.75% and 3%, ATC concentrations between 0.01 and 0.04 mg./ml., and freezing, thawing, or storing plasma at 25° for up to 43 hr. appeared to have very little effect on the catalytic potency of human plasma with respect to ATC hydrolysis.

4. The catalytic activity of human plasma appeared to be concentrated in the III-0, IV-4, and V Cohn fractions. The activity of Fraction V was probably due to contaminating enzymes from the other fractions, and inhibition studies with physostigmine and TEPP suggested that the activities of Fractions III-0 and IV-4 were due to pseudocholinesterase.

5. Human and horse pseudocholinesterases and the chymotrypsins were the most potent catalysts of ATC hydrolysis

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IV.

Interaction of Carboxypeptidase A with Carbamate and Carbonate Esters†

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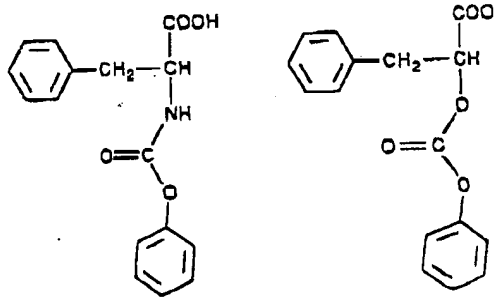
ABSTRACT: The carbamate ester *N*-(phenoxycarbonyl)-L-phenylalanine binds well to carboxypeptidase A in the manner of peptide substrates. The ester exhibits linear competitive inhibition toward carboxypeptidase A catalyzed hydrolysis of the amide hippuryl-L-phenylalanine ($K_i = 1.0 \times 10^{-3}$ M at pH 7.5) and linear noncompetitive inhibition toward hydrolysis of the specific ester substrate *O*-hippuryl-L-β-phenyllactate ($K_i = 1.4 \times 10^{-3}$ M at pH 7.5). Linear inhibition shows that only one molecule of inhibitor is bound per active site at pH 7.5. The hydrolysis of the carbamate ester is not affected by the presence of 10^{-8}–10^{-9} M enzyme (the concentrations employed in inhibition experiments), but at an enzyme concentration of 3×10^{-6} M catalysis can be detected. The value of k_{cat} at 30 °C, $\mu = 0.5$ M, and pH 7.45 is 0.25 s^{-1}, and K_m is 1.5×10^{-3} M. The near identity of K_m and K_i shows that K_m is a dissociation constant. Substrate inhibition can be detected at pH less than 7 but not at pH values above 7, which suggests that a conformational change is occurring near that pH. The analogous carbonate ester *O*-(phenoxycarbonyl)-L-β-phenyllactic acid is also a substrate for the enzyme. The K_m is pH independent from pH 6.5 to 9 and has the value of 7.6×10^{-5} M in that pH region. The rate constant k_{cat} is pH independent from pH 8 to 10 at 30 °C ($\mu = 0.5$ M) with a limiting value of 1.60 s^{-1}. Modification of the carboxyl group of glutamic acid-270 to the methoxyamide strongly inhibits the hydrolysis of *O*-(phenoxycarbonyl)-L-β-phenyllactic acid. Binding of β-phenyllactate esters and phenylalanine amides must occur in different subsites, but the ratios of k_{cat} and k_{cat}/K_m for the structural change from hippuryl to phenoxy in each series are closely similar, which suggests that the rate-determining steps are mechanistically similar.

Carboxypeptidase A (peptidyl L-amino acid hydrolase, EC 3.4.12.2) is a Zn(II) metalloenzyme that catalyzes the hydrolysis of peptides and *O*-acyl derivatives of α-hydroxycarboxylic acids (Hartsuck & Lipscomb, 1971). From X-ray crystallographic studies of the enzyme in the presence of poor peptide substrates it has been presumed that the metal ion complexes the substrate carbonyl oxygen. Glutamic acid-270 has also been implicated in the catalytic reaction, and both nucleophilic and general base mechanisms have been suggested involving carboxyl group participation (Lipscomb, 1970; Ludwig & Lipscomb, 1973; Kaiser & Kaiser, 1972).

Kinetic evidence (biphasic kinetics) has been presented for the formation of an intermediate in the hydrolysis of *O*-(*trans*-*p*-chlorocinnamoyl)-L-β-phenyllactate and the corresponding *p*-dimethylamino-substituted ester at subzero temperature in a mixed aqueous–organic solvent system (Makinen et al., 1976; Hoffman et al., 1983). However, there does not appear to be any spectral evidence for buildup of an anhydride even at low temperature (Hoffman et al., 1983). Experiments designed to detect the presence of an anhydride intermediate in ester or peptide hydrolysis in H_2O at normal temperatures have failed to do so (Kaiser & Kaiser, 1972; Breslow & Wernick, 1977; Breslow et al., 1983; Galdes et al., 1983). Rapid breakdown of a reactive anhydride through reversal of the acylation reaction and hydrolysis would preclude direct observation of the intermediate under normal conditions of temperature and solvent with conventional types of ester and amide substrates. Furthermore, experiments with added alcohol nucleophiles, in which evidence for transesterification is sought, have an inherent ambiguity when specific substrates for carboxypeptidase A are employed in that the leaving group will bind strongly in the active site and might not depart until the anhydride hydrolyzes (Hoffman et al., 1983). Thus, it is not known whether an anhydride is a general intermediate in ester and peptide hydrolysis, and as a consequence, it has often not been clear which step in the reaction was being monitored in kinetic studies.

The carbamate and carbonate esters I and II possess a terminal aromatic side chain and carboxyl group that are



I II

necessary for binding (Hartsuck & Lipscomb, 1971), and the phenolic leaving groups would permit the enzyme reaction to be easily monitored spectrophotometrically. Carbonate diesters have not been previously investigated as possible substrates for carboxypeptidase A. Carbamate esters have been extensively studied as inhibitors of bovine pancreatic carboxypeptidase A (CPA)[1] (Awazu et al., 1967; Auld & Vallee, 1970a; Kaiser et al., 1965), but compounds with phenolic leaving groups of this type have not been previously employed

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[1] Abbreviations: CPA, bovine pancreatic carboxypeptidase A; HPL, *O*-hippuryl-L-β-phenyllactic acid; HPA, hippuryl-L-phenylalanine; Cbz-Gly, carbobenzoxyglycine; CPL, *O*-(*trans*-cinnamoyl)-L-β-phenyllactic acid; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)-aminomethane.

in kinetic studies. Expulsion of phenol would be expected to occur rapidly in an intracomplex nucleophilic reaction of these compounds. Neighboring alkoxide (Hutchins & Fife, 1973a), phenoxide (Hutchins & Fife, 1973b), amino, sulfhydryl (Fife et al., 1975), and carboxylate (Hegarty et al., 1974) groups have been shown to be highly effective nucleophiles in intramolecular reactions of carbamate esters, and various intramolecular nucleophilic groups are effective in reactions of carbonate diesters (Fife & Hutchins, 1981). The phenol group of I and II should also depart readily from the active site, thereby minimizing reversibility of the reaction in comparison with reactions of corresponding carboxylate derivatives. Therefore, in view of the hydrolytic lability of anhydrides (Fife & Przystas, 1983), nucleophilic attack on such compounds should be the rate-determining step and might be studied without complication from the possible anhydride breakdown. Consequently, compounds of this general type could have considerable mechanistic utility.

Experimental Procedures

Materials. N-(Phenoxycarbonyl)-L-phenylalanine (I) was prepared by refluxing 2 equiv of L-phenylalanine (Sigma) and 1 equiv of phenyl chloroformate (Eastman) for 48 h in chloroform that was washed and dried by the method of Perrin et al. (1966). The mixture was then filtered. The chloroform was removed by rotary evaporation, and the remaining solid was recrystallized from dry benzene. The white needles were subsequently vacuum dried over calcium sulfate. The compound had mp 62–63 °C. Anal. Calcd for $C_{16}H_{15}NO_4$: C, 67.36; H, 5.30; N, 4.91. Found: C, 66.97; H, 5.42; N, 4.73.

O-(Phenoxycarbonyl)-L-β-phenyllactic Acid (II). Phenyl chloroformate (2.69 g, 0.017 mol), in 100 mL of diethyl ether was added to a solution of L-β-phenyllactic acid (2.8 g, 0.017 mol) in 150 mL of diethyl ether. The mixture was stirred in an ice bath for 15 min. Pyridine (1.4 mL, 0.017 mol) in 150 mL of diethyl ether was then added dropwise over a period of 3 h. The reaction mixture was allowed to stir for 16 h. The mixture was then filtered to remove pyridine hydrochloride, and the filtrate was rotary evaporated. The residual oil was dissolved in 10 mL of dichloromethane and applied to a 2.5 × 30 cm silica gel column equilibrated with 99% dichloromethane–1% methanol. Fifty-milliliter fractions were collected at a flow rate of 3 mL/min. Column fractions were analyzed by silica gel thin-layer chromatography, and the components were identified by comparison with standards. With this column technique, the carbonate diester II was successfully separated from contaminating phenol, L-β-phenyllactic acid, and other possible products (e.g., the anhydride formed upon reaction of phenyl chloroformate with the carboxyl group of L-β-phenyllactic acid). Column fractions containing II were combined, rotary evaporated, placed under a vacuum for 16 h, and stored at −20 °C in a desiccator. The final product was a clear oil at room temperature that only solidified at −20 °C. Attempted distillation of the oil resulted in decomposition. The TLC of II showed only one spot. Attempts to prepare the sodium salt and dicyclohexylammonium salt of II were unsuccessful. Complete hydrolysis of II gave a quantitative release of 1 equiv of phenol. Titration of the carboxyl group of II and phenol release in hydrolysis gave quantitatively equivalent results; i.e., the equivalents of OH^- consumed in titration equaled the equivalents of phenol released in hydrolysis. Therefore, the chromatographically pure material is free of all evident impurities.

O-Hippuryl-L-β-phenyllactic acid (HPL) was obtained as the sodium salt from Vega-Fox Biochemicals, and hippuryl-

Table I: Spectral Data Employed in Kinetic Studies[a]

compd	pH	wavelength (nm)	ε (M^{-1} cm^{-1})
hippurate	7.50	254	2600
	5.53	254	2750
I	6.0–10	270	180
phenol + phenylalanine	7.50	270	1300
	5.63	270	1250
II	6.0–10	270	202
phenol + phenyllactate	6.0–10	270	1230

[a] T = 30 °C (μ = 0.5 M, NaCl).

Co. *O-(trans*-Cinnamoyl)-L-β-phenyllactate (CPL) was prepared by reaction of distilled cinnamoyl chloride with β-phenyllactic acid as described by Hall et al. (1969). Formation of the sodium salt is described by King and Fife (1983). Carboxypeptidase A was obtained from Sigma (carboxypeptidase A–DFP from bovine pancreas). The buffers employed were all of reagent grade. Reagent sodium chloride (Mallinckrodt) was used to maintain ionic strength. Deionized water was used throughout.

Stock solutions of HPL sodium salt were prepared by using deionized water. Spectroscopic grade (Matheson) acetonitrile was used to prepare the stock solutions of substrate esters I and II. The HPA stock solutions were also made with acetonitrile. The concentration of these solutions was adjusted so that the added acetonitrile represented 1% or less of the total reaction solution volume (3 mL per cuvette). Initial velocity measurements were the same in the absence or presence of this amount of acetonitrile. Enzyme stock solutions were prepared in a cold room at 4 °C as described previously (Kaiser, 1970). The commercial enzyme suspension was added to an appropriate amount of cold Tris buffer (0.05 M, 0.5 M NaCl, pH 7.50) and dialyzed against three successive changes of fresh buffer solution. After centrifugation the supernatant solution was stored at 4 °C. The enzyme concentration was determined spectrophotometrically, $\epsilon_{278} = 6.42 \times 10^4$ M^{-1} cm^{-1} (Simpson et al., 1963).

Kinetic Methods. Initial velocity measurements were carried out with a Beckman Model 25 or Pye Unicam SP8-100 recording spectrophotometer according to the procedure of Hall et al. (1969). Temperature was maintained at 30 ± 0.1 °C. An appropriate aliquot of enzyme stock solution was added to a cuvette containing 2–3 mL of buffer (0.05 M, μ = 0.5 M with NaCl) and allowed to thermally equilibrate. A predetermined aliquot of inhibitor and/or substrate was then added, and the reaction was monitored at a selected wavelength. The absorbance due to the enzyme was negligible at the concentrations employed (<3.0 μM). The molar extinction coefficients used to monitor the enzymatic reactions are presented in Table I. Initial velocity determinations in the reaction of 2×10^{-9} M CPA with 1×10^{-4} M HPL and/or CPL at pH 7.5 were reproducible and were conducted at the beginning and end of each set of experiments. The enzyme catalyzed hydrolysis of I and II was monitored by following an increase in absorbance at 270 nm due to phenol. Initial rate data were always corrected for spontaneous hydrolysis, but such correction was of negligible significance in the reactions of I at pH <9. Reaction solution pH values were measured with a Radiometer Model 22 or a Beckman Model 3500 pH meter that had been standardized with Mallinckrodt standard buffer solutions. The buffers employed were Tris–acetate (pH 6.50–7.0), Tris-HCl (pH 7.0–8.5), and Ammediol–HCl (pH 9.0–9.5).

Formation of Methoxyamide Carboxypeptidase A. The

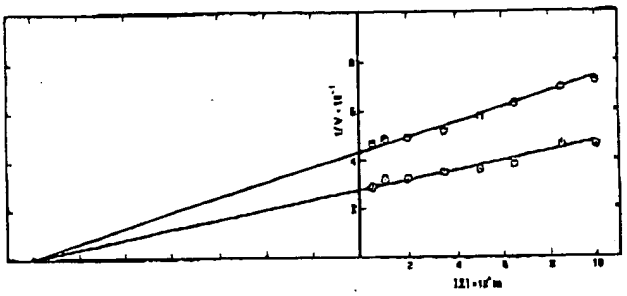


FIGURE 1: Plots of $1/V$ vs. *N*-(phenoxycarbonyl)-L-phenylalanine (I) concentration for reaction of 1×10^{-4} M (●) or 5×10^{-4} M (⊙) hippuryl-L-β-phenyllactate with carboxypeptidase A at 30 °C. Velocity is expressed as the change in concentration of product per minute measured at 254 nm. The pH was 7.5 (0.05 M Tris and μ = 0.5 M with NaCl).

peptidase A by *N*-ethyl-5-phenylisoxazolium-3′-sulfonate (Woodwards reagent K) and subsequent formation of the methoxyamide derivative was that of Petra (1971) with the modifications described in King and Fife (1983). In kinetic studies with the modified enzyme, assay buffers were 0.05 M with an ionic strength of 1.0 M with NaCl. Enzyme concentrations were varied from 4×10^{-9} M to 8×10^{-6} M depending on the substrate.

RESULTS

The nonenzymatic rates of hydrolysis of *N*-(phenoxycarbonyl)-L-phenylalanine (I) were too slow to be accurately measured at 30 °C at pH values close to neutrality. Rate constants were obtained at pH >10. The reactions are OH^- catalyzed with $k_{OH} = 2.68\ M^{-1}\ s^{-1}$. The nonenzymatic release of phenol from *O*-(phenoxycarbonyl)-L-β-phenyllactic acid (II) in 50% dioxane–H_2O (v/v) at pH <10 exhibits a k_{obsd} vs. pH profile at 30 °C that is sigmoidal with $pK_{app} = 4.3$. Titration of II in the same solvent yielded a pK_a value of 4.4. The pH-independent portion of the profile has a limiting rate constant of $9.01 \times 10^{-4}\ s^{-1}$. The D_2O solvent isotope effect in this region (k_{H_2O}/k_{D_2O}) is 1.03.

Plots of $1/V$ vs. $1/(S)_0$ for carboxypeptidase A catalyzed hydrolysis of *O*-hippuryl-L-β-phenyllactic acid (HPL) in the presence of three constant concentrations of I and in the absence of I at pH 7.5 and 30 °C were linear and intersected on the $1/(S)_0$ axis. In the absence of I, K_m has the value of 8.1×10^{-5} M and k_{cat} is 460 s^{-1}. The K_m is in reasonable agreement with the value of 1×10^{-4} M obtained from the data of Bunting et al. (1974). A plot of $1/V$ vs. the concentration of I is shown in Figure 1. Linear noncompetitive inhibition is indicated with a K_i value of 1.4×10^{-3} M. Carboxypeptidase A catalyzed hydrolysis of HPL is known to be subject to substrate inhibition (Bunting et al., 1974). In all experiments substrate concentrations less than 0.001 M were therefore employed.

Plots of $1/V$ vs. $1/(S)_0$ for carboxypeptidase A catalyzed hydrolysis of hippuryl-L-phenylalanine (HPA) in the presence of three constant concentrations of I and in the absence of I at pH 7.5 and 30 °C were linear and intersected on the vertical axis, which indicates competitive inhibition. The value of k_{cat} is 100 s^{-1} and K_m is 1.7×10^{-3} M in the absence of inhibitor, which compares well with values found previously (Davies et al., 1968b). The linear plot of $1/V$ vs. the concentration of I is shown in Figure 2 from which a K_i value of 1×10^{-3} M was obtained. A horizontal line through the point of intersection of the lines strikes the vertical axis at the value of $1/V_{max}$ determined from the plots of $1/V$ vs. $1/(S)_0$. Substrate activation has been previously observed for carboxypeptidase

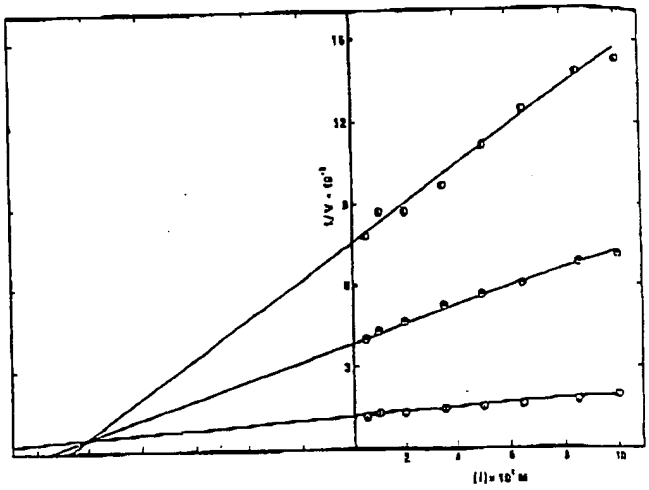


FIGURE 2: Plots of $1/V$ vs. *N*-(phenoxycarbonyl)-L-phenylalanine (I) concentration for reaction of 5×10^{-5} (◐), 1×10^{-4} (●), and 5×10^{-4} M (⊙) hippuryl-L-phenylalanine with carboxypeptidase A at 30 °C. Velocity is expressed as the change in concentration of product per minute measured at 254 nm. The pH was 7.5 (0.05 M Tris and μ = 0.5 M with NaCl).

Table II: Values of k_{cat} and K_m for CPA-Catalyzed Hydrolysis of *N*-(Phenoxycarbonyl)-L-phenylalanine (I) and *O*-(Phenoxycarbonyl)-L-β-phenyllactic Acid (II) at 30 °C

compd	pH	k_{cat} (s^{-1})	$K_m \times 10^3$ (M)	k_{cat}/K_m ($M^{-1}\ s^{-1}$)
I	6.50	0.018	0.147	120
	6.93	0.042	0.265	158
	7.45	0.25	1.53	166
	7.95	0.28	1.75	162
	8.49	0.28	1.91	146
	8.99	0.18	2.05	86
	9.39	0.14	3.01	47
II	6.50	0.45	0.075	6000
	7.00	0.73	0.076	9600
	7.50	1.02	0.075	13600
	8.00	1.25	0.085	14700
	8.50	1.59	0.077	20650
	9.00	1.50	0.080	18750
	9.50	1.54	0.089	17300
	9.98	1.60	0.149	10740

however, no such effects were observed in this study at the concentrations employed.

Although CPA catalysis of the rate of phenol release from *N*-(phenoxycarbonyl)-L-phenylalanine (I) was not detected at enzyme concentrations of 10^{-8}–10^{-9} M (the concentrations in inhibition experiments of Figures 1 and 2), at the higher enzyme concentration of 3×10^{-6} M a catalytic reaction could be observed. Nicely linear plots of V vs. $V/(S)_0$ were obtained at pH values greater than 7.0 from which the values of k_{cat} and K_m in Table II were obtained. Note that the K_m at pH 7.5 is similar to the K_i obtained in the inhibition experiments. The values of k_{cat} are a bell-shaped function of pH with a maximum at pH 8 (see Table II). At pH <7 there was marked downward curvature in the plots of V vs. $V/(S)_0$ at substrate concentrations greater than 10^{-3} M, which indicates substrate inhibition, in contrast with the linearity of such plots at pH >7. The values of k_{cat} and K_m at pH 6.93 and 6.50 were therefore determined by employing data obtained at substrate concentrations less than 10^{-3} M. In the plot of log k_{cat}/K_m vs. pH (not shown), there is a clearly defined pK_2^E of 8.8 ± 0.1.

The dicarbonate ester II is also a substrate for CPA. A plot of velocity vs. enzyme concentration for the hydrolysis of 7.87×10^{-5} M II at pH 7.5 with the CPA concentration being

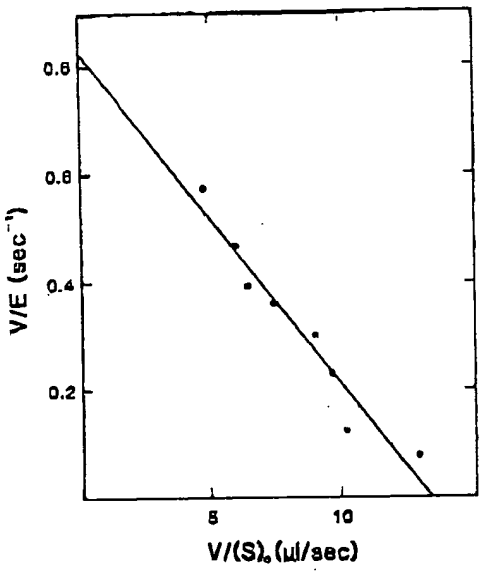


FIGURE 3: Typical V/E vs. $V/(S)_0$ plot for the carboxypeptidase A catalyzed hydrolysis of *O*-(phenoxycarbonyl)-L-β-phenyllactic acid at 30 °C and pH 7.00 (0.05 M Tris–0.5 M NaCl).

Table III: Glutamic Acid-270 Modification

enzyme	k_{cat} (s^{-1}) (pH 7.5), CPL	% control	k_{cat} (s^{-1}) (pH 7.5), II	% control
native	155	100	1.24	100
methoxyamide CPA + inhibitor[a]	109	70.3	0.88	71.0
methoxyamide CPA[b]	11.7	7.5	0.08	6.5

[a] Enzyme preincubated with β-phenylpropionic acid was treated with Woodwards reagent K and the methoxyamine according to the method of Petra (1971). [b] The enzyme was treated with Woodwards reagent K followed by conversion to the methoxyamide according to the method of Petra (1971).

to zero enzyme concentration slightly above the origin. This indicated that nonenzymatic hydrolysis is affecting the velocity by <10%. The velocities were, however, corrected for this spontaneous hydrolysis. Enzymatic rate constants for the hydrolysis of II were calculated from V/E vs. $V/(S)_0$ plots of initial rate data, of which Figure 3 is a typical example. Values of k_{cat} and K_m are given in Table II; k_{cat} is pH independent at pH >8. The limiting k_{cat} value is 1.60 s^{-1}. The K_m is pH independent in the range 6.5–9 but with gentle increases at the extremes of both low and high pH. The pH-independent value of K_m is 7.6×10^{-5} M. The log k_{cat}/K_m vs. pH profile is bell-shaped with values of 6.9 and 9.6 ± 0.2 for pK_1^E and pK_2^E, respectively. Initial velocity plots of absorbance vs. time were linear even at very high concentrations of enzyme (2×10^{-5} M) and $(S)_0 \gg (E)_0$ in reactions of both I and II at pH 7.5.[2]

The carboxyl group of Glu-270 was modified to the methoxyamide by the method of Petra (1971) employing *N*-ethyl-5-phenylisoxazolium-3′-sulfonate (Woodwards reagent K) followed by treatment with methoxyamine. This modified enzyme is inactive toward II at pH 7.50 as shown by the data in Table III. Prior incubation of the enzyme with the inhibitor β-phenylpropionic acid at a concentration of 4 mM protects the enzyme against inactivation by Woodwards reagent K and methoxyamine, thereby showing that the active site is being modified.

Discussion

Carbamate Ester Inhibition of Carboxypeptidase A. The carbamate ester I possesses the required structural features for strong binding to carboxypeptidase A and must be binding in the site for peptide substrates. Compound I competitively inhibits the CPA-catalyzed hydrolysis of hippuryl-L-phenylalanine ($K_i = 1.0 \times 10^{-3}$ M at pH 7.5) but exhibits noncompetitive inhibition toward hydrolysis of hippuryl-L-β-phenyllactic acid ($K_i = 1.4 \times 10^{-3}$ M at pH 7.5), which has been classified as a specific ester substrate (Bunting et al., 1974). The K_i value of 1.4×10^{-3} M for I shows that it binds to the enzyme much more strongly than the carbamate ester carbobenzoxyglycine (Cbz-Gly) for which K_i values of 2.9×10^{-2} M and 1.6×10^{-2} M have been found for inhibition of the hydrolysis of Cbz-Gly-Gly-L-Phe (Auld & Vallee, 1970a) and *O*-(*trans*-cinnamoyl)-DL-β-phenyllactate (Awazu et al., 1967), respectively. *N*-Carbobenzoxy-L-phenylalanine is a competitive inhibitor toward Cbz-Gly-Gly-L-Phe with $K_i = 6 \times 10^{-4}$ M (Byers & Wolfenden, 1973).

When the active site of an enzyme is large and a relatively small substrate or inhibitor is being studied, caution must be employed in the interpretation of results since binding might then occur in a nonspecific manner. Kinetic anomalies such as substrate activation or substrate inhibition, implying multiple binding modes, have frequently been associated with binding of small molecules to CPA. Acylated tripeptides, on the other hand, behave more simply than shorter homologues (Auld & Vallee, 1970a). The interaction of carbobenzoxyglycine with CPA gives rise to competitive inhibition toward tripeptides (Auld & Vallee, 1970a) and the specific ester substrate CPL (Awazu et al., 1967). In the CPA-catalyzed hydrolysis of dipeptides it behaves as an activator (Davies et al., 1968), and in the hydrolysis of hippurylglycolate, a nonspecific ester substrate, Cbz-Gly acts to suppress substrate activation (Kaiser et al., 1965). It should be noted that Cbz-Gly does not possess an aromatic R group in the amino acid portion of the molecule, a preferred feature for strong binding to CPA (Ludwig & Lipscomb, 1973). Thus, it is not surprising that unusual inhibition and activation effects are observed. In contrast, I does not give rise to such abnormal effects at pH 7.5.

The linear inhibition plots of Figures 1 and 2 indicate that one inhibitor molecule is binding per active site at pH 7.5. Binding of more than one inhibitor molecule or both inhibitor and amide substrate simultaneously [see Figure 12 of Ludwig and Lipscomb (1973)] would give rise to nonlinear $1/V$ vs. (I) plots. Partial competitive inhibition in which an EIS complex is formed will give linear $1/V$ vs. $1/(S)_0$ plots but curvature in the $1/V$ vs. (I) plots (Segel, 1975). Linear mixed-type inhibition can be obtained in cases where an EIS complex is totally unreactive, but then both V_{max} and K_m will be affected; i.e., the plots of $1/V$ vs. $1/(S)_0$ would intersect behind the vertical axis (Segel, 1975). Thus, an EIS complex is not formed in reactions of HPA, and it is therefore reasonable to conclude that I is binding to the active site in a similar or identical manner as specific amide and peptide derivatives of L-phenylalanine. It has been suggested that ester and peptide substrates might bind in separate but adjoining sites on the enzyme (Bunting & Kabir, 1977; Auld & Holmquist, 1974). In those studies carboxylic acid inhibitors were found to be competitive vs. esters and noncompetitive vs. am-

[2] Only a small absorbance change would be associated with an initial burst in these reactions of I and II. However, biphasic kinetics were also

by I in the hydrolysis of HPA and HPL but from the reverse standpoint; i.e., I is competitive vs. amides and noncompetitive vs. esters. Unlike carboxylic acid inhibitors, I also exhibits substrate properties; therefore, it is clear that the inhibitor's effect is due to binding in a catalytically important site.

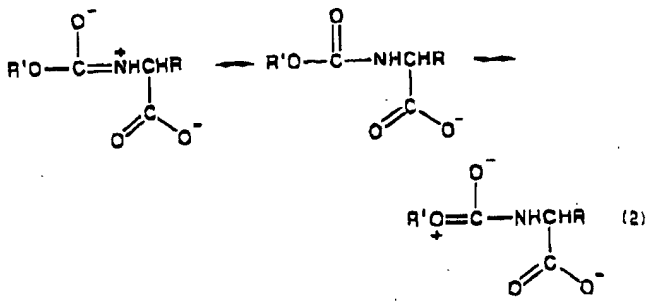
At high enzyme concentration (3×10^{-6} M) CPA catalysis of the release of phenol from the carbamate ester I can be readily detected. The K_m of 10^{-3} M for I compares favorably with the K_m values (pH 7.5) for many of the best amide and peptide substrates. For example, K_m for benzoyl-Gly-Gly-Phe is ca. 10^{-3} M (Auld & Vallee, 1970a) as is that determined for hippuryl-L-phenylalanine in this work. Results have been presented that are compatible with K_m being a measure of the peptide substrates's binding affinity (Auld & Vallee, 1970a,b). Since $K_m = K_i$ in the hydrolysis of I, it is clear that $K_m = K_s$; i.e., K_m represents the dissociation constant of the enzyme–substrate complex. Consequently, if an anhydride intermediate is produced with initial phenol release, as in eq 1, its formation

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \text{ (anhydride)} \xrightarrow{k_3} RCOO^- + E + \text{(phenoxide)} \quad (1)$$

(k_2) must be rate determining. Phenol must bind weakly to CPA since it is a rather poor inhibitor of reactions catalyzed by the enzyme; 50% inhibition in the hydrolysis of 10^{-3} M HPL is achieved by a 0.05 M concentration (Davies et al., 1968a). The reaction of eq 1 would therefore be essentially irreversible, and K_m would be $K_s k_3/k_2$ if anhydride hydrolysis was rate determining. Only if the carbamate nitrogen was protonated in a tetrahedral intermediate might phenylalanine be the initial leaving group. Such a process does not occur in the chemical intramolecular reactions of phenolic carbamate esters (Hutchins & Fife, 1973a,b; Hegarty et al., 1974); phenol is the leaving group in those reactions. However, the decline in k_{cat} with increasing pH suggests that an acidic group is involved in the CPA-catalyzed hydrolysis of I. The values of K_m and K_s would also be equal if the reaction proceeds without the formation of an intermediate.

Even though the phenoxy group of I would provide a good leaving group, the value of k_{cat} is relatively small (approximately 500-fold less than the k_{cat} values of tripeptide substrates and 400-fold less than those of amide substrates such as hippuryl-L-phenylalanine). Larger substrates are characterized by relatively large k_{cat} values. This may be due to a better steric fit to functional groups in the active site, although the increased possibilities for binding contacts do not result in appreciably lower K_m values (Auld & Vallee, 1970a; Ludwig & Lipscomb, 1973). Thus, binding energy may be expended to produce a more favorable rate. A twisting effect aided by multiple binding contacts would reduce the resonance interaction between the amide nitrogen and the carbonyl and thereby make the carbonyl group more susceptible to nucleophilic attack. Such an effect would be especially difficult with the carbamate I because of the large resonance interaction (eq 2) and the small size of the substrate. Thus, the k_{cat} values of I may in part demonstrate the importance of this resonance effect; i.e., $K_m(K_s)$ is normal but k_{cat} is small.

The k_{cat} values for I decline as pH is decreased below 7. This is also the case in the plot of log k_{cat}/K_m vs. pH (pK_a^E values of 6.7 and 8.8). Thus, as with other substrates for CPA there is an important apparent pK_a near 7. The occurrence of substrate inhibition at pH less than 7 (such inhibition is not observed at pH >7) indicates that a conformational change



R = benzyl; R' = phenyl (2)

permits binding of I in an additional site. Nonproductive binding in an additional site would lower k_{cat} and K_m identically (Fersht, 1977). Note in Table II that at pH 6.93 both k_{cat} and K_m are substantially less than at higher pH but that k_{cat}/K_m remains nearly unchanged. Nonproductive binding might not occur with large substrates, but the conformation change at pH <7 could still be important catalytically if it also occurs in the ES complex and could influence the apparent pK_a^{ES} of 6–7. Thus, a pH-dependent conformational change of the type that is very likely occurring in the reaction of I could be of general significance in CPA-catalyzed reactions.

CPA-Catalyzed Hydrolysis of O-(Phenoxycarbonyl)-L-β-phenyllactate. The carbonate diester II is also a substrate for carboxypeptidase A. The value of K_m (pH 6.5–9) is 7.6×10^{-5} M, which is slightly less than that of the specific ester substrate *O*-(*trans*-cinnamoyl)-L-β-phenyllactic acid (1.9×10^{-4} M) (Hall et al., 1969). The values of pK_1^E and pK_2^E of 6.9 and 9.6 obtained from the plot of log (k_{cat}/K_m) vs. pH are also comparable to those found for CPL (6.5 and 9.4). Thus, II is very likely binding in the active site in the same manner as specific esters. Phenol release in the hydrolytic reaction is quantitative, and all of the phenol is released in a single rapid reaction. However, k_{cat} values for II are 100-fold less than those obtained for hydrolysis of CPL in the pH range 6.5–9 and 400-fold less than in the hydrolysis of HPL at pH 7.5. The value of k_{cat}/K_m at pH 7.5 for II is 1.4×10^4 M^{-1} s^{-1} as compared with 1.7×10^2 M^{-1} s^{-1} in the case of I. Therefore, the carbonate ester is a better substrate than the analogous carbamate. The ratio k_{cat}/K_m is the second-order rate constant for reaction of the free enzyme with substrate and is unaffected by any nonproductive binding of the substrate. This constant is therefore the most reliable parameter to employ in assessing relative substrate ability (Fersht, 1977). The difference in substrate ability for I and II resides largely in the less favorable K_m value of I. The difference in the k_{cat} values for I and II at pH 7.5 is only a factor of 4, which is similar to the 5-fold difference in k_{cat} for HPA and HPL. The difference in k_{cat}/K_m for I and II (82-fold) is likewise very similar to that for HPA and HPL (100-fold).

The values of k_{cat} for CPA-catalyzed hydrolysis of II are pH independent in the pH range 8–10 and decline with decreasing pH below pH 8. The hydrolysis of CPL is characterized by a k_{cat} vs. pH profile that is sigmoidal below pH 9 but with a rapidly rising arm at pH >9 (Hall et al., 1969; King & Fife, 1983). It will be noted that such an apparent OH^--catalyzed reaction does not occur in hydrolysis of II at pH values below 10. It has been considered that breakdown of an anhydride intermediate is the rate-determining step in the CPA-catalyzed hydrolysis of β-phenyllactate esters (Makinen et al., 1979; Kaiser & Kaiser, 1972), and the increasing rate constants with increasing pH at pH >9 are in accord with Zn(II)-promoted attack of OH^- on an anhydride. In contrast, the pH independence of the k_{cat} values of II at

mediate or the substrate unless the pK_a of metal ion liganded water is abnormally low [see Discussion in King and Fife (1983)].

Modification of the carboxyl group of Glu-270 to the methoxyamide by the method of Petra (1971) greatly inhibits activity toward *O*-(phenoxycarbonyl)-L-β-phenyllactate just as it does toward CPL (King & Fife, 1983). Thus it is clear that an intact carboxyl group (or carboxylate anion) is necessary for enzyme catalysis. A simple interpretation is that Glu-270 is directly involved in the catalytic process either as a nucleophile or as a general base, but it is, of course, also possible that the carboxyl group of Glu-270 is structurally necessary to maintain the active site in the proper conformation.

A nucleophilic mechanism might be anticipated in the hydrolysis of II, considering the possibility of phenol expulsion. It is chemically reasonable that phenol would be the initial leaving group in such a reaction because of the large difference in the pK_a values of phenol (10) and the β-phenyllactate alcohol group (14.7) estimated by the method of Fox and Jencks (1974). If nucleophilic attack by Glu-270 were occurring in that manner, then as in the case of I, formation of the anhydride should be rate determining because of the reduced tendency of phenol to bind in the active site. Rate-determining anhydride breakdown would not be expected unless reversibility occurs, as in eq 3, with $k_{-2} > k_3$. L-β-Phenyllactate, initially

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \underset{\overset{|}{ROH}}{ES'\ (\text{anhydride})} \xrightarrow{k_3} RCOO^- + E \quad (3)$$

liberated in the hydrolysis of CPL, will, on the other hand, bind strongly in the active site ($K_i = 5.8 \times 10^{-5}$ M at pH 7.5) (Hall et al., 1969) presumably in position to reverse the reaction (Hall & Kaiser, 1967; Kaiser & Kaiser, 1972). In view of the much lower pK_a of phenol than β-phenyllactate (4.7 pK_a units), expulsion of the latter from a tetrahedral intermediate in preference to the former would require a strained C–O bond or proton transfer to the leaving group. Since k_{cat} is pH independent at pH >8, such a proton transfer might only occur from water and would take place most readily if water were the nucleophile adding to the carbonyl group.

The relatively small values of k_{cat} and k_{cat}/K_m for I and II are significant in view of the much greater reactivity of those compounds in nonenzymatic hydrolysis reactions than carboxylic ester and amide substrates for CPA. For example, the second-order rate constant k_{OH} for OH^--catalyzed hydrolysis of CPL in only 0.017 M^{-1} s^{-1} at 30 °C (King & Fife, 1983). Therefore, the high nonenzymatic hydrolytic reactivity of I and II due to the phenolic leaving group is not manifested in the k_{cat} and k_{cat}/K_m values. As discussed, the small k_{cat} values could reflect an imprecise alignment in the active site. However, the K_m values are normal and give no evidence of unusual binding. The rate of anhydride formation should be markedly enhanced by a significant reduction of the leaving group pK_a. Consequently, the small k_{cat} values in combination with the absence of burst kinetics at high enzyme concentrations[2] and the relationship $K_m = K_i$ (with I) indicate that a nucleophilic reaction in which phenol is the leaving group cannot be a favorable process. The effect of the phenoxy group with both I and II may be due mainly to its relatively small size and perhaps in part to its inductive electron-withdrawing effect. Such an effect should decrease the facility of breakdown of a tetrahedral intermediate to give β-phenyllactic acid or phenylalanine.

Rate-Determining Step. From X-ray crystallographic

Table IV: Values of k_{cat}/K_m at pH 7.5 for Ester and Amide Substrates for CPA

compd	k_{cat}/K_m (M^{-1} s^{-1})	ratio (ester/amide)
(1) II	1.4×10^4	82
I	1.7×10^2	
(2) HPL	$5.7 \times 10^{6\,a}$	97[a]
	$6.6 \times 10^{6\,b}$	60[b]
	$5.3 \times 10^{6\,c}$	
HPA	$5.9 \times 10^{4\,a}$	
	$1.1 \times 10^{5\,b}$	
(3) Bz-Gly-L-OPhe	$1.5 \times 10^{6\,d}$	75
Bz-Gly-L-Phe	$2 \times 10^{4\,d}$	
(4) Dns-Gly-L-OPhe	$7.3 \times 10^{6\,d}$	84
Dns-Gly-L-Phe	$8.7 \times 10^{4\,d}$	
(5) Bz-Gly$_3$-L-OPhe	$1.5 \times 10^{6\,d}$	125
Bz-Gly$_3$-L-Phe	$1.2 \times 10^{4\,d}$	

[a] This work, 30 °C. [b] Davies et al. (1968b), 25 °C. [c] Bunting et al. (1974), 25 °C, 0.2 M NaCl. [d] Auld & Holmquist (1974), 25 °C, 1.0 M NaCl.

analogous to esters (O replaced by $-CH_2$), Rees and Lipscomb (1981) and Lipscomb (1980) have suggested that the catalytic site for both esters and peptides is the same and is that in which the aromatic residue can fit in the hydrophobic pocket and the terminal carboxylate group interacts with Arg-145. The different inhibition patterns of esters and peptides (Auld & Holmquist, 1974), which had been explained on the basis of different sites for the two types of substrates, were then attributed to different rate-determining steps. Hydrolysis of esters was considered to involve rate-determining breakdown in the catalytic site S_1', whereas the rate-determining step with peptides was suggested to be movement from the subsite S_2, near Phe-279, Tyr-198, and Arg-71, into S_1' [see also Cleland (1977)]. The greater difficulty of binding peptides than esters in S_1' was thought to reside in the twisting effect necessary to achieve binding to the metal ion and the hydrophobic pocket and to allow hydrogen bonding with Tyr-248. As noted, resonance in a carbamate ester (eq 2) would be especially pronounced, and therefore twisting the molecule would be difficult. However, a structural change from phenylalanine to β-phenyllactic acid produces the same relative change in k_{cat} and k_{cat}/K_m for the phenoxy and hippuryl derivatives. Likewise, a structural change from hippuryl to phenoxy produces the same change in k_{cat}/K_m in each series (4×10^2). This would not be expected if association with the catalytic site was rate determining for amides but not esters, considering the differences in size and reactivity of these compounds. A chemical step is very likely rate limiting with both types of substrates.

A similar analysis of the data of Auld and Holmquist (1974) on the CPA-catalyzed hydrolysis of oligopeptides and depsipeptides again reveals that the ratios of k_{cat}/K_m for ester–amide pairs having phenyllactate or phenylalanine as the terminal group are not greatly dependent on structure, the ratios varying from 75 to 125 (Table IV). Thus, through a wide variation in structure, the ratio of k_{cat}/K_m for an ester to k_{cat}/K_m for the analogous amide is nearly independent of structure with large specific substrates and the small nonspecific substrates I and II. The ratio is apparently larger with a cinnamoyl acyl group (4720) (McClure & Neurath, 1966; Kaiser & Kaiser, 1972) because the amide is an especially poor substrate, but the ratio is 185 with the α-(acetylamino)cinnamoyl derivatives and 111 with the α-naphthoylamino-substituted compounds (Suh et al., 1985).

Esters and amides must bind in different subsites. Since

pH range, it is indeed probable that the catalytic reactions of I and II are also occurring in subsites that are different. That does not, however, preclude the possibility of common features in the mechanisms if substrate-dependent conformational changes of the enzyme are occurring.

A plausible scheme would involve initial formation of ES followed by a conformational change in which the catalytic site is formed. This would then be followed by breakdown of the substrate, which may or may not involve Glu-270. The fact that I is a linear noncompetitive inhibitor toward esters (HPL) very likely means that a conformational change takes place due to its binding (or that one necessary for the reaction of esters is prevented) so that the EIS complex does not break down. Two intermediates have been detected in the hydrolysis of both peptide and ester substrates prior to the rate-determining step (Galdes et al., 1983; Geoghegan et al., 1983). The above scheme provides for at least two intermediates before the product-forming step ($ES_1 \rightleftharpoons ES_2$).

Registry No. I, 56379-89-6; II, 104070-51-1; HPL, 3675-74-9; HPA, 744-59-2; CPA, 11075-17-5; L-Glu, 56-86-0; PhO_2CCl, 1885-14-9; L-$PhCH_2CH(OH)CO_2H$, 20312-36-1.

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METABOLISM OF BAMBUTEROL IN RAT LIVER MICROSOMES: IDENTIFICATION OF HYDROXYLATED AND DEMETHYLATED PRODUCTS BY LIQUID CHROMATOGRAPHY MASS SPECTROMETRY

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ABSTRACT:

The oxidative metabolism of (*R,S*)-bambuterol in rat liver microsomes was studied. Metabolite fractions were analyzed by thermospray LC-MS. The use of an equimolar mixture of deuterium-labeled and unlabeled bambuterol facilitated the mass spectrometric identification of the metabolites. Six metabolites, formed via hydroxylation, demethylation, and hydrolytic reactions, were identified. The demethylated metabolites were found to be chemically unstable under physiological conditions. It is likely that the complex biotransformation of bambuterol into terbutaline is one factor contributing to the long duration of action of bambuterol.

(*R,S*)-Bambuterol[1] is a bis-dimethylcarbamate prodrug of the bronchodilator terbutaline (1) (fig. 1). Clinical studies have shown that bambuterol administration gives prolonged duration of therapeutic effects and reduces adverse symptoms when compared with administration of terbutaline itself (2, 3). Prolonged absorption of bambuterol and slow formation of terbutaline allows once-daily dosage of the drug in the treatment of asthma (4). The advantageous properties of bambuterol are probably in part dependent on the features of the bambuterol biotransformation. Terbutaline can be formed from bambuterol by a two-step hydrolysis via the monocarbamate derivative (D2439; see fig. 1), a reaction catalyzed predominantly by plasma cholinesterase (5). This enzyme is selectively inhibited by bambuterol (6), *i.e.* the prodrug inhibits its own hydrolysis. Preliminary experiments with tritiated bambuterol incubated with rat liver microsomes showed that several other metabolites were formed, probably by oxidative reactions. The purpose of the present study was to isolate and identify the major microsomal metabolites of bambuterol. Identification was accomplished by on-line thermospray LC-MS (7). This technique permitted the use of reversed phase LC systems for separation of the metabolites and afforded mild conditions for the vaporization and ionization of the thermally labile molecules. Deuterium-labeled analogues were used to create artificial isotope clusters in the mass spectra of the metabolites, thereby facilitating identification.

Materials and Methods

Nomenclature. Synthetic reference compounds (fig. 1) are referred to by their generic names (terbutaline and bambuterol) or by their code number (*e.g.* D2439). Metabolite fractions are referred to by roman numerals. Roman numerals with index *a* or *b* are used to designate the hydroxylated and demethylated metabolites, respectively, in the metabolite fractions.

[1] Throughout the text bambuterol refers to the racemic drug. All reference compounds were prepared as the racemates. The stereochemical configurations of the metabolites are not known.

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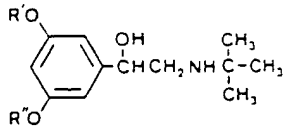
Chemicals. Bambuterol, terbutaline, D2439, D2467, D2468, and D2469 (see fig. 1) were synthesized as the racemates at the Organic Chemistry Laboratory, AB Draco (Lund, Sweden) (8). [$^2H_6$]Bambuterol and [$^2H_6$]D2439 were prepared by similar methods, using deuterium-labeled *t*-butylamine. The latter compound was prepared from [$^2H_6$] acetone, via a Grignard reaction to produce *t*-[$^2H_6$]butanol, which was subsequently transferred via a Ritter reaction into the desired *t*-[$^2H_6$] butylamine.

Tritiated bambuterol ([^{3}H]bambuterol; 1-[3,5-bis(*N,N*-dimethylcarbamoyloxy)phenyl]-2-*t*-butylamino[1-^{3}H]ethanol hydrochloride), with a specific activity of 0.97 Ci/mmol, was obtained from the Radiochemical Centre (Amersham, UK). The radiochemical purity was 97% as determined by LC (LC system A; all LC systems are described in table 1). Other chemicals were of analytical grade and purchased from commercial sources. All water used was purified in a Milli-Q system (Millipore, Molsheim, France).

Rat Liver Microsomes. Male Sprague-Dawley rats (200–225 g) were treated with phenobarbital (ip 80 mg/kg once a day) for 3 days and killed on day 4. The livers were homogenized in 4 volumes of ice-cold 50 mM Tris-HCl buffer, pH 7.5, containing 5 mM $MgCl_2$ and 0.25 M sucrose. The homogenate was centrifuged at 4°C at 9000*g* for 15 min, and thereafter the supernatant fraction was centrifuged at 4°C at 100,000*g* for 90 min. The microsomal pellet was then suspended in the same buffer, yielding a protein concentration of 21.6 mg/ml. The microsomal suspension was kept frozen at −20°C in aliquots of 0.7 ml until use.

Incubations. The overall pattern of bambuterol metabolites formed by rat liver microsomes was obtained by incubating [^{3}H]bambuterol at a concentration of 96 nM for 90 min. The pattern of radioactive metabolites was used to determine the collection time intervals for the various fractions collected for metabolite identification in other experiments. All incubations were performed at 37°C in 50 mM potassium phosphate buffer, pH 7.5, at a protein concentration of 2 mg/ml. NADPH, when used, was added at a concentration of 2 mM. Metabolite fraction II was prepared for mass spectrometric identification by incubating an equimolar mixture of bambuterol and [$^2H_6$]bambuterol at a total drug concentration of 0.5 mM for 1 hr. Metabolite fraction V was prepared from a similar incubation, starting with a mixture of D2439 and [$^2H_6$] D2439. Metabolite fraction IV was prepared by isolation and further incubation of metabolite fraction II, obtained as described above.

Extraction of Metabolites. After incubation the metabolites were extracted on Sep-Pak C_{18} cartridges (Waters Associates, Milford, MA). The cartridge was conditioned by rinsing it three times with 3 ml of ethanol, twice with 3 ml of water, and once with 3 ml of 10 mM



	R′	R″
Bambuterol	CH_3, CH_3 >NCO	CH_3, CH_3 >NCO
D2439	CH_3, CH_3 >NCO	H
D2467	H, CH_3 >NCO	H, CH_3 >NCO
D2468	H, CH_3 >NCO	H
D2469	CH_3, CH_3 >NCO	H, CH_3 >NCO
Terbutaline	H	H

FIG. 1. *Chemical structures of synthetic reference compounds.*

phosphate buffer, pH 7.5. The incubation mixture was then passed through the cartridge, followed by two 3-ml portions of water. The bambuterol metabolites were eluted with two 1-ml portions of ethanol containing 5% of 50 mM ammonium chloride buffer, pH 8.5.

Isolation of Metabolite Fractions. The eluates from the Sep-Pak cartridges were evaporated to dryness under nitrogen at 60°C. Each residue was dissolved in 0.5 ml of the initial mobile phase of the gradient LC system to be used. System A (see table 1) was used for isolation of metabolite fraction V and system B for isolation of metabolite fractions II and IV. Two hundred microliters were injected into the LC system and 0.5-min fractions collected with a Gilson fraction collector. The metabolite-containing fractions were stored at −20°C until analyzed by LC-MS.

Mass Spectrometry. A Finnigan thermospray LC-MS interface was used with a Finnigan 4500 mass spectrometer connected to an Incos data system (Finnigan MAT, San José, CA). LC system C was used for analysis of metabolite fractions II and IV. The vaporizer temperature was set at 112°C, the jet temperature at 180°C, and the repeller potential at 190 V. LC system D was used for analysis of metabolite fraction V, and the corresponding MS settings were: vaporizer, 160°C; jet, 200°C; and repeller, 160 V. Mass spectra of metabolite fractions II and IV were acquired from m/z 150 to 500 at a rate of 2 sec per scan. For metabolite fraction V the scan range was m/z 200–400 and the rate 1 sec per scan.

Measurement of Radioactivity. Radioactivity was determined in a Packard TriCarb Scintillation Spectrometer. Ten milliliters of Optifluor (Packard Instrument Co., Downers, Grove, IL) were added to each fraction.

Stability Test. The chemical stability of the N-monomethylcarbamates D2467, D2468, and D2469 (fig. 1) at pH 7.5 and 5.0 was investigated. Solutions (0.3 mM) of the compounds were prepared in 0.1 M potassium phosphate buffer, pH 7.5, or 0.1 M ammonium acetate buffer, pH 5.0. Both solutions were incubated at room temperature and the solution prepared in phosphate buffer, pH 7.5, also at 37°C. Samples were taken at different time intervals during 24 hr. Before analysis, aliquots of 0.5 ml were mixed with 0.5 ml of an internal standard solution prepared in the same buffer and at about the same concentration as the compound of interest. Solutions of D2467 and D2469 were analyzed on LC system E (see table 1) with bambuterol as internal standard, and solutions of D2468 were analyzed on LC system F with D2439 as internal standard.

TABLE 1

Liquid chromatography systems

System A	Column: Nucleosil C_{18}, 5 μm (Macherey-Nagel), 150 × 4.6 mm Mobile phase A: 0.05 M ammonium acetate buffer, pH 4.6 Mobile phase B: 10% 0.5 M ammonium acetate buffer, pH 4.6, 90% methanol Gradient: linear program from 20% B to 50% B during 10 min; during next 10 min linear program to 90% B Flow rate: 1.0 ml/min Injector: Waters WISP 710B automatic injector Pumps: 2 Waters M45 Gradient controller: Waters automatic gradient controller Detector: Waters M440 UV detector (254 nm)
System B	Column: Nucleosil 10SA (Macherey-Nagel), 150 × 5 mm Mobile phase A: 0.25 M ammonium acetate buffer, pH 4.6 Mobile phase B: 50% 0.5 M ammonium acetate buffer, pH 4.6, 50% acetonitrile Gradient: linear program from 10% B to 90% B during 20 min Flow rate: 1.0 ml/min Instrumentation: same as for LC system A
System C	Column: Nucleosil 10SA (Macherey-Nagel), 150 × 5 mm Mobile phase: 10% 1 M ammonium acetate buffer, pH 5.2, 48% water, 42% methanol Flow rate: 1.4 ml/min Injector: Valco model C6W manual injector with a model A60 air actuator Pump: LKB 2150 Detector: Waters M440 UV detector (254 nm) connected in line with the mass spectrometer
System D	Column: Nucleosil C_{18}, 5 μm (Macherey-Nagel), 150 × 5 mm Mobile phase: 10% 1 M ammonium acetate buffer, pH 5.2, 70% water, 20% methanol Flow rate: 1.4 ml/min Instrumentation: same as for LC system C
System E	Column: Nucleosil C_{18}, 5 μm (Macherey-Nagel), 100 × 3 mm Mobile phase: 65% 0.1 M ammonium acetate buffer, pH 5.0, 35% methanol Flow rate: 0.4 ml/min Injector: Varian 9090 automatic injector Pump: Waters M590 Detector: Waters M490 UV detector (254 nm)
System F	Same as system E, except for the composition of the mobile phase, which was 75% 0.1 M ammonium acetate buffer, pH 5.0, and 25% methanol.

Results

Typical retention times of the various bambuterol metabolites and synthetic reference compounds in the LC systems used are given in table 2. The retention times varied slightly over the experimental period but reference compounds were used to characterize the chromatographic systems on each occasion. No

TABLE 2

Typical retention times of metabolites and synthetic reference compounds, and collection intervals for metabolite fractions in LC systems A–F

Compound	Mol wt	LC retention time (min)					
		A	B	C	D	E	F
Bambuterol	367	18.0	16.5			9.7	
Hydroxylated bambuterol (IIa)	383	14–16	14.5–15.5	15.6			
Demethylated bambuterol	353						
D2469		15.2	14.9	17.6		4.6	
IIb		14–16	14.5–15.5	17.6			
Monocarbamate (D2439)	296	13.7	13.1	12.2			7.6
Dihydroxylated bambuterol (IVa)	399	10.5–12.5	12.5–14	8.3			
Hydroxylated-demethylated bambuterol (IVb)	369	10.5–12.5	12.5–14	9.2			
Didemethylated bambuterol (D2467)	339	11.5	13.1	10.4		2.7	
Hydroxylated monocarbamate (Va)	312	8.5–10	11–12		15.6		
Demethylated monocarbamate	282						
D2468		9.0	11.3		11.0		3.1
Vb		8.5–10	11–12		11.0		
Terbutaline	225	6.3	9.5				

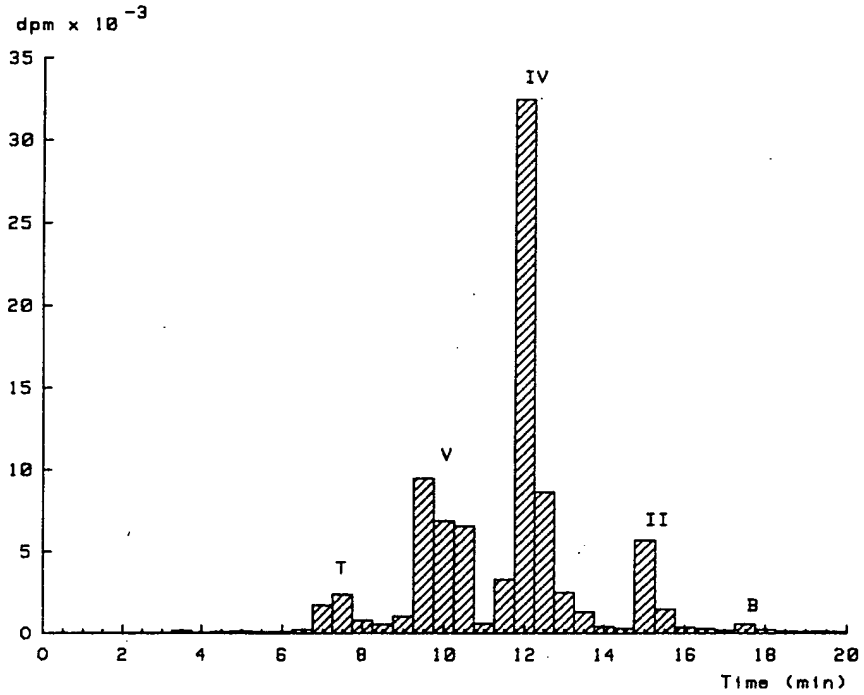


FIG. 2. *Radiochromatogram obtained after incubation of [^{3}H]bambuterol with rat liver microsomes.*

LC system A was used and 0.5-min fractions collected. The retention time of the monocarbamate (D2439) under these conditions was 13.7 min. *T*, terbutaline; *B*, bambuterol.

significant separation of the deuterium-labeled analogues from the unlabeled compounds was observed.

Metabolite Pattern. Fig. 2 shows the radiochromatogram obtained after incubation of [^{3}H]bambuterol with liver microsomes from phenobarbital-treated rats in the presence of NADPH. A relatively low substrate concentration (96 nM) was chosen to facilitate the formation of secondary and tertiary metabolites. Three metabolites or groups of metabolites were detected, apart from terbutaline and D2439. These metabolite fractions are called II, IV, and V. Under the conditions chosen (LC system A), there was a tendency of metabolite fraction V to be split into a double peak. The investigations presented below demonstrated that this fraction, as well as metabolite fractions II and IV, contained at least two components. In the absence of NADPH, very little biotransformation occurred. The only reaction observed was the formation of small amounts of the monocarbamate (2–3% of the substrate).

Other studies, which will be reported separately, have demonstrated that metabolite fraction II is a primary product of bambuterol, that metabolite fraction IV is a product of fraction II, and that metabolite fraction V can be formed from either D2439, fraction II, or fraction IV. Thus, metabolite fractions for analysis by LC-MS were prepared by stepwise incubations as described under *Materials and Methods*.

Analysis of Metabolite Fraction II. LC-MS analysis of metabolite fraction II (LC system C) revealed two chromatographic

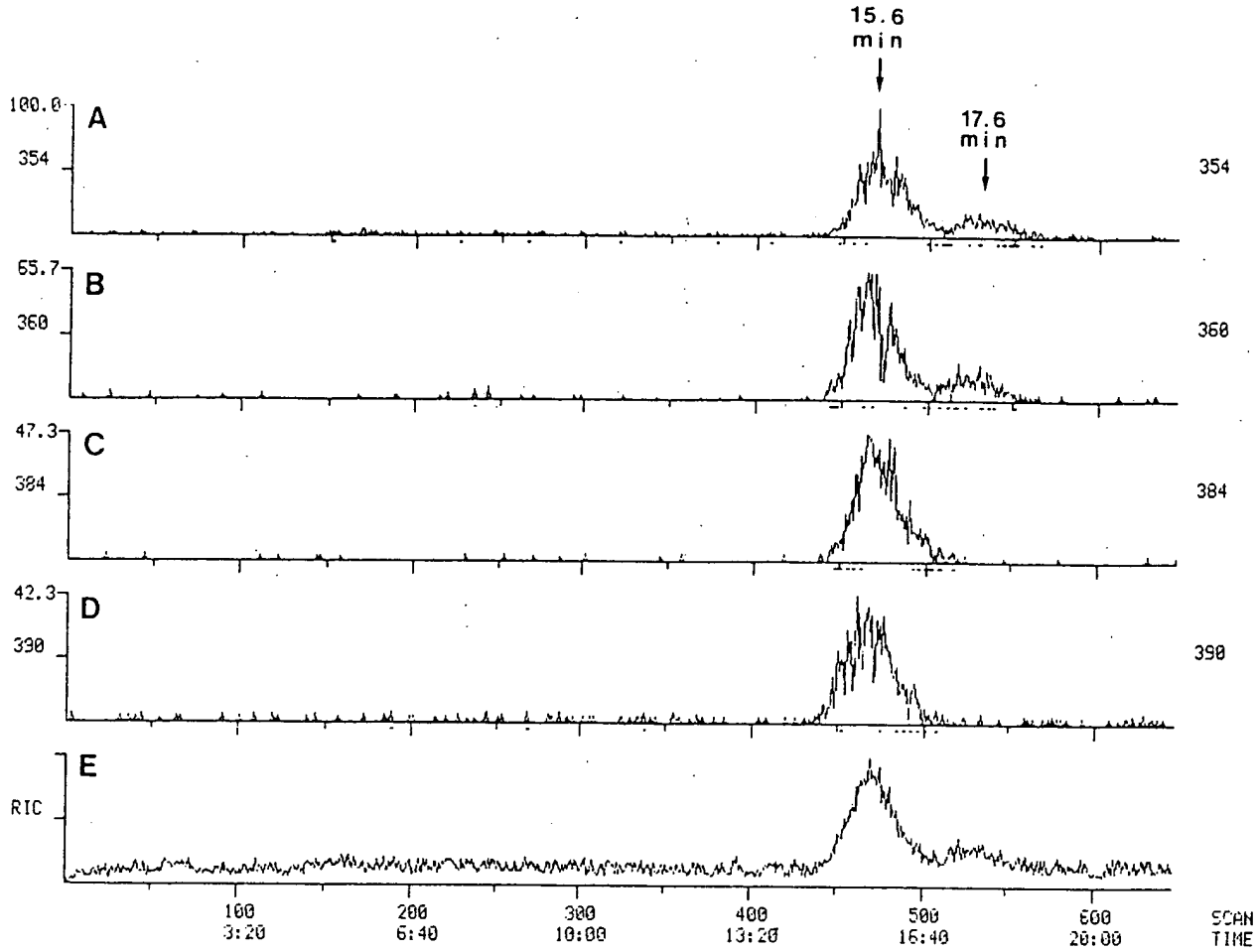


FIG. 3. *Total ion current (E) and selected ion current (A-D) chromatograms from LC-MS analysis of metabolite fraction II (LC system C).*

peaks (fig. 3). The smaller peak had a retention time of 17.6 min, which was identical to that of D2469 (fig. 1), *i.e.* demethylated bambuterol. The mass spectrum of this metabolite (fig. 4*B*) showed peaks at *m/z* 354 and 360, which correspond to the MH^+ ions of demethylated bambuterol and its deuterium-labeled analogue. The base peaks at *m/z* 297 and 303 are probably formed by loss of the demethylated carbamate side chain from the MH^+ ions (MH^+—CH_3NCO). The mass spectrum of D2469 (fig. 4*A*) was almost identical and confirmed the identity of the metabolite as demethylated bambuterol.

Fig. 4*C* shows the mass spectrum of the larger chromatographic peak (fig. 3) with a retention time of 15.6 min. The peaks at *m/z* 384 and 390 correspond to the MH^+ ions of hydroxylated bambuterol and its deuterated analogue. Loss of formaldehyde from the MH^+ ions can explain the peaks at *m/z* 354 and 360, and loss of CH_3NCO in a second step results in the ions at *m/z* 297 and 303. It was recently reported that loss of formaldehyde from a hydroxymethyl amide metabolite of zolpidem occurred during the thermospray process (9).

Analysis of Metabolite Fraction IV. Fig. 5 shows chromatograms from an LC-MS analysis of metabolite fraction IV (LC system C). The selected ion current chromatograms (fig. 5, *A* and *B*) indicate that the fraction contains two metabolites that can decompose to terbutaline (*m/z* 226) and deuterated terbutaline (*m/z* 232) during the mass spectrometric evaporation/ionization process. The mass spectrum of the first component (R_T = 8.3 min) showed peaks at *m/z* 400 and 406 (fig. 6*A*), corresponding to the MH^+ ions of dihydroxylated bambuterol and its deuterium-labeled analogue. The peaks in the mass spectrum can be rationalized as outlined in the fragmentation scheme in fig. 7. The fragmentation shows that the two hydroxyl groups must be situated in separate carbamate groups.

Fig. 6*B* shows the mass spectrum of the second component with a retention time of 9.2 min. The peaks at *m/z* 370 and 376 suggest that this compound is hydroxylated-demethylated bambuterol/[$^2H_6$]bambuterol. The spectrum resembles very closely the spectrum of dihydroxylated bambuterol (fig. 6*A*), and starting with the ion at *m/z* 370 the fragmentation scheme in fig. 7 can be used for interpretation. The mass spectrum is consistent with a structure in which one carbamate group is hydroxylated and the other one is demethylated. Didemethylated bambuterol was available as reference (D2467; R_T = 10.4 min) but could not be identified as a metabolite in the incubation mixture.

Fig. 5, *C* and *D*, shows the presence of the monocarbamate of bambuterol (*m/z* 297 and 303; R_T = 12.0 min), probably as a result of incomplete separation during fractionation. Another metabolite with a mass spectrum similar to that of D2439 was found at a retention time of 10.3 min. The identity of this metabolite is unknown but a possible structure is discussed below.

Analysis of Metabolite Fraction V. Fig. 8 shows the total ion current chromatogram from an LC-MS analysis of metabolite fraction V (LC system D). The retention time of the smaller peak (11.0 min) was identical to that of demethylated monocarbamate

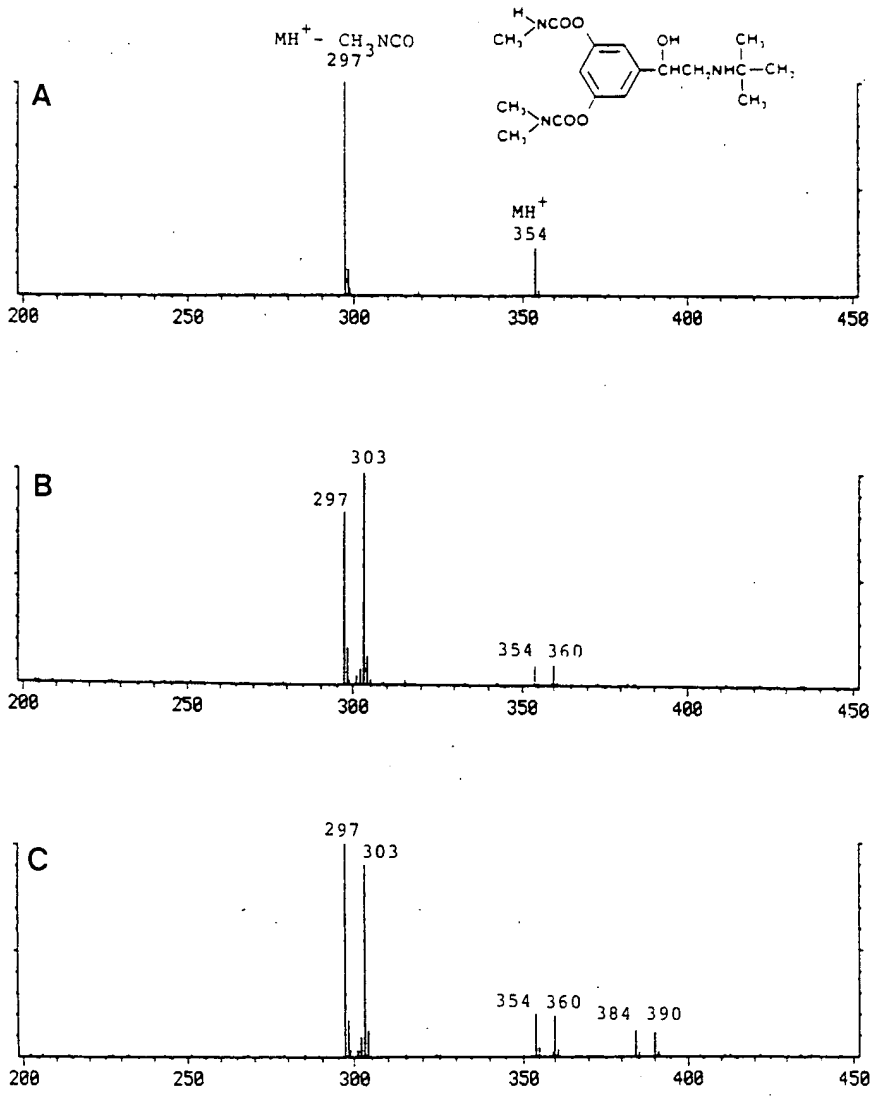


FIG. 4. *TSP mass spectra of compounds related to metabolite fraction II (cf. Fig. 3).*

A, authentic D2469; *B*, compound with $R_T = 17.6$ min (demethylated bambuterol); *C*, compound with $R_T = 15.6$ min (hydroxylated bambuterol).

(D2468). The mass spectra of the metabolite and the reference compound were similar (fig. 9, *A* and *B*).

The mass spectrum of the larger peak ($R_T = 15.6$ min) showed a pair of ions at *m/z* 313 and 319 (fig. 9*C*), corresponding to the MH^+ ions of hydroxylated monocarbamate and its deuterated analogue. The ions at *m/z* 283 and 289 are probably formed by loss of formaldehyde and the ions at *m/z* 226 and 232 by subsequent loss of CH_3NCO.

Chemical Stability of *N*-Monomethylcarbamates. The *N*-monomethylcarbamates proved to be unstable at pH 7.5 (fig. 10), decomposing by loss of the monomethylcarbamate side chain. The half-lives of D2467, D2468, and D2469 at 37°C were 0.97, 7.0, and 1.7 hr, respectively. At room temperature the half-lives were about five to six times longer. No decrease in concentration was observed for any of the compounds in buffer of pH 5.0 after incubation at room temperature for 24 hr.

Discussion

The oxidative metabolism of bambuterol in rat liver microsomes is most likely similar to that of a general *N*-demethylation reaction (10), *i.e.* hydroxylation followed by elimination of formaldehyde from the intermediate carbinolamine. Because hydroxylation of bambuterol takes place at a carbamate moiety, a much more stable carbinolamide is formed, permitting its isolation and identification. Relatively stable hydroxylated metabolites have also been reported to be formed from other carbamates (11, 12) and amides (9, 13). Our studies do not answer the question of whether the release of formaldehyde from the hydroxylated metabolites is enzymatically catalyzed or whether it is a nonenzymatic decomposition. Of interest in this connection is a report indicating the existence of an enzyme in rat intestine microsomes catalyzing the release of formaldehyde from *N*-hydroxymethylpentamethylmelamine (14). In another investigation, *N*-formylbenzamide was proposed to be a chemically unstable metabolite of the stable *N*-hydroxymethylbenzamide (15). It is possible that *N*-formyl derivatives could be formed from the various demethylated metabolites of bambuterol. No such *N*-formyl metabolites were identified but the hypothetical *N*-formyl derivative of demethylated monocarbamate would give the same molecular weight as the monocarbamate and could thus explain the unknown metabolite with a retention time of 10.3 min in fig. 5.

Once formed, the demethylated metabolites are chemically

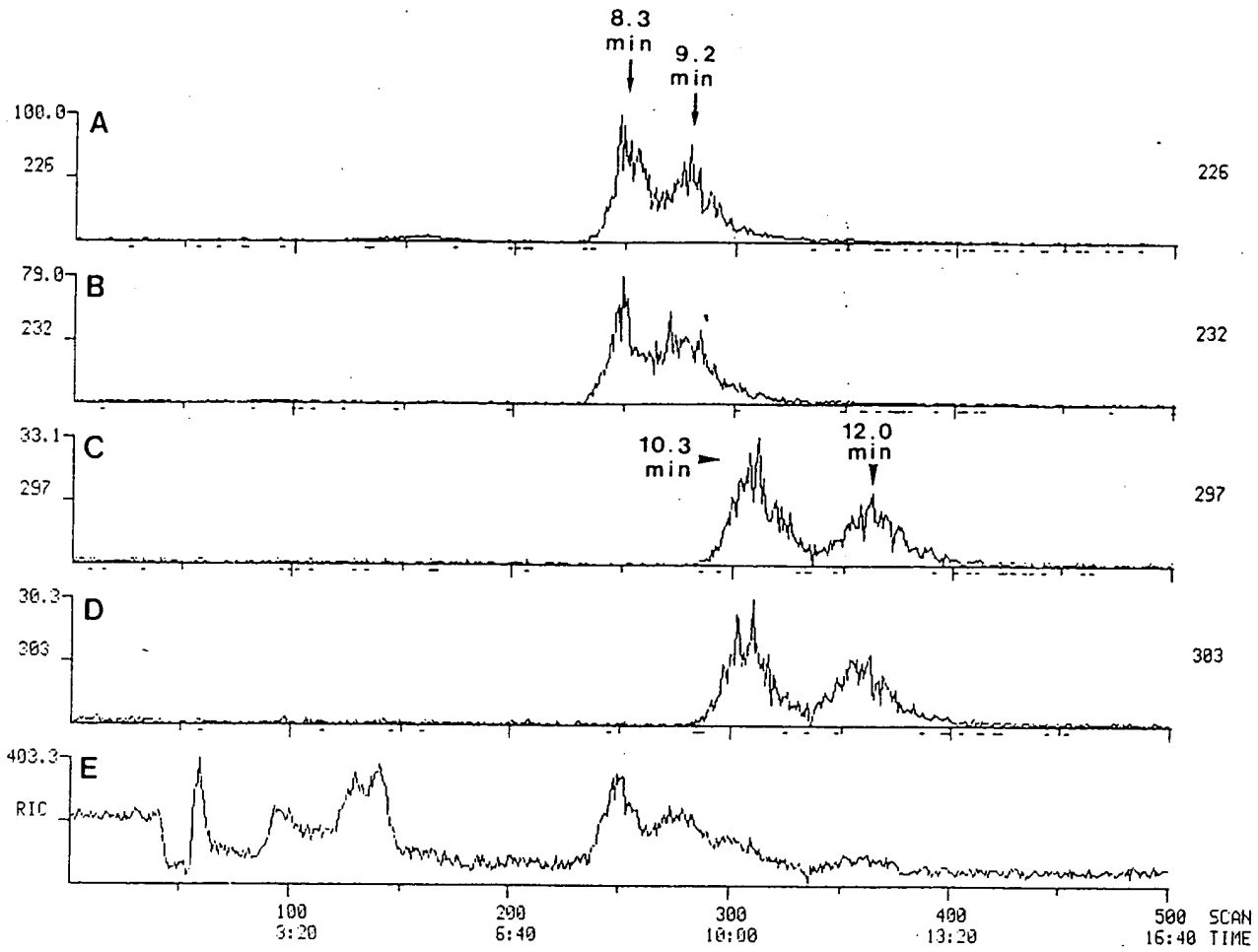


FIG. 5. *Total ion current (E) and selected ion current (A–D) chromatograms from LC-MS analysis of metabolite fraction IV (LC system C).*

unstable at physiological pH, as shown in fig. 10. Whereas bambuterol and other phenyl-*N,N*-dimethylcarbamates are stable in neutral aqueous solution, the corresponding *N*-monomethyl derivatives are usually found to be about 10^6 times less stable (16). The reason for this difference in stability is that *N*-monomethylcarbamates decompose via an ElcB mechanism, whereas *N,N*-dimethylcarbamates are hydrolyzed via the common ester $B_{Ac}2$ mechanism. Didemethylated bambuterol (D2467) was the most labile compound ($t_{1/2}$ = 0.97 hr), which is probably the reason that it could not be identified as a metabolite of bambuterol. Hitherto, we have been unable to identify metabolites formed from the hydroxylated or demethylated compounds by a second oxidative reaction at the same carbamate group.

In summary, we have identified seven metabolic intermediates (including D2439) between bambuterol and terbutaline. These intermediates are results of hydroxylation, demethylation, and hydrolytic reactions. Thus, a complex series of pathways for the formation of terbutaline from bambuterol can be proposed (fig. 11). It should be pointed out that considerable species differences exist regarding the balance between oxidative and hydrolytic biotransformation of bambuterol. For example, the hydrolytic activity of rat liver microsomes is low compared with that of human liver microsomes.[2] It is likely that the complexity of the metabolism is one factor contributing to the long duration of the therapeutic effects of bambuterol.

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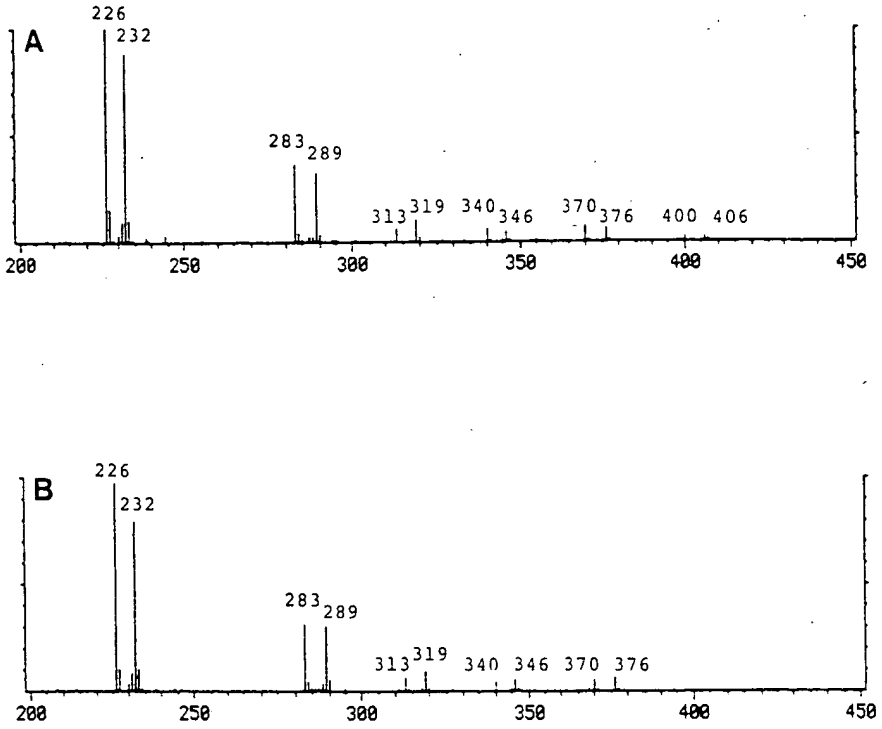

FIG. 6. *TSP mass spectra of compounds related to metabolite fraction IV (cf. Fig. 5).*

A, compound with $R_T = 8.3$ min (dihydroxylated bambuterol); *B*, compound with $R_T = 9.2$ min (hydroxylated-demethylated bambuterol).

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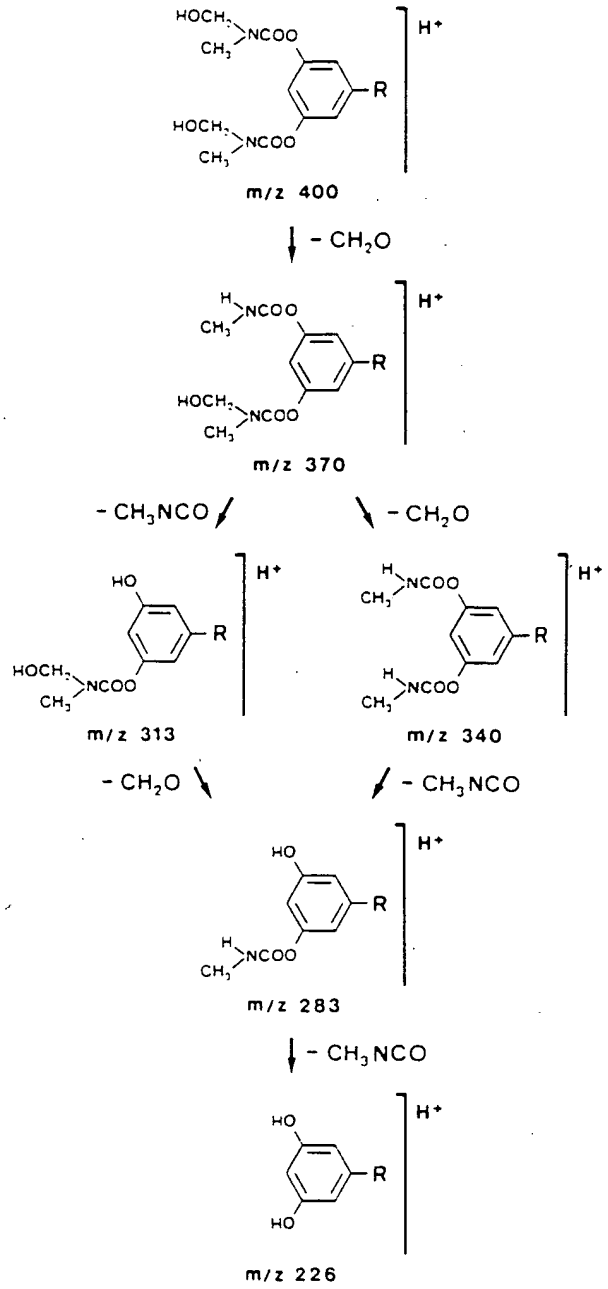

FIG. 7. *Mass spectrometric fragmentation of dihydroxylated bambuterol (IVa) (cf. Fig. 6A).*

$R = CH(OH)CH_2NHC(CH_3)_3$

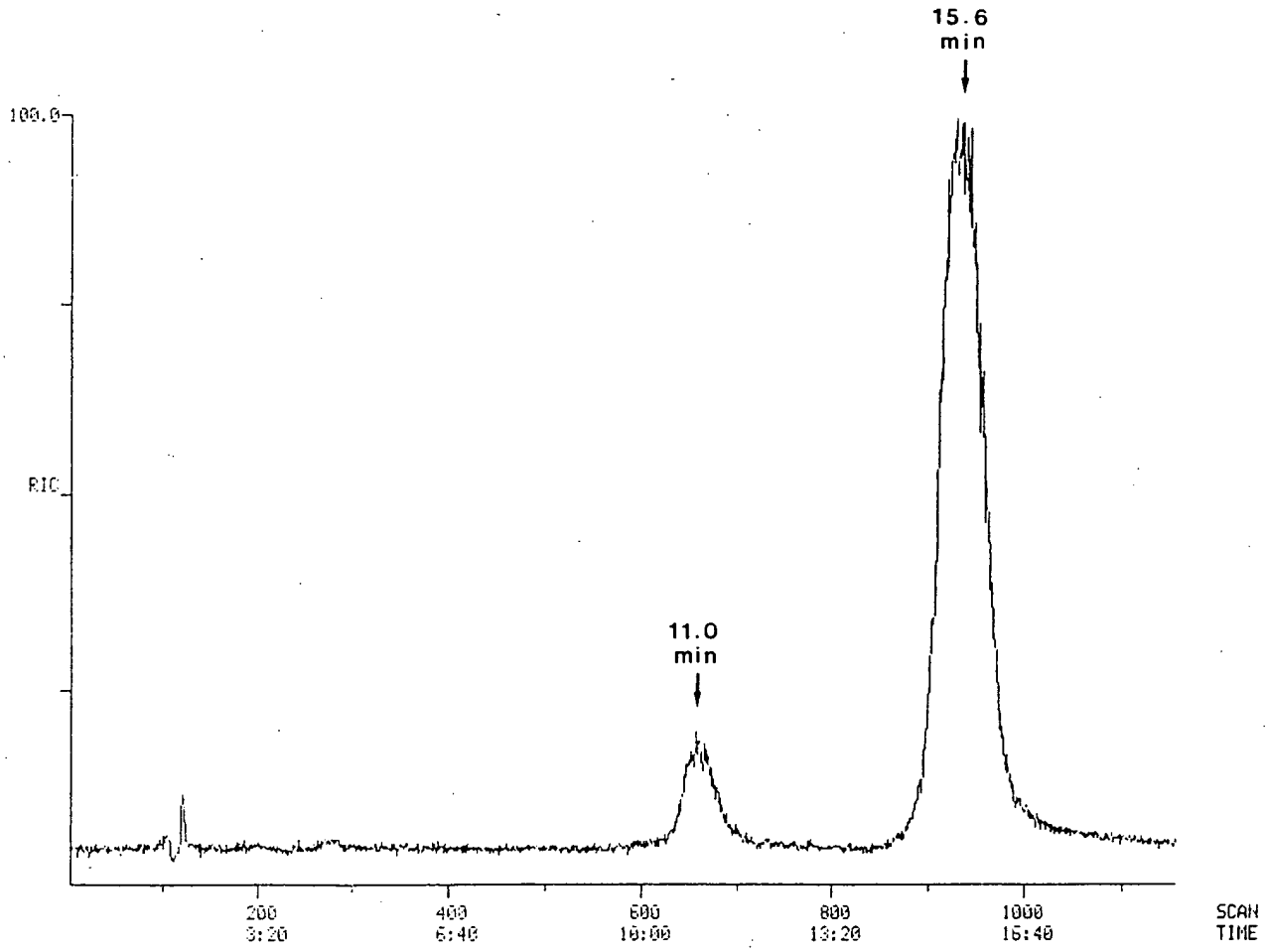

FIG. 8. *Total ion current chromatogram from LC-MS analysis of metabolite fraction V (LC system D).*

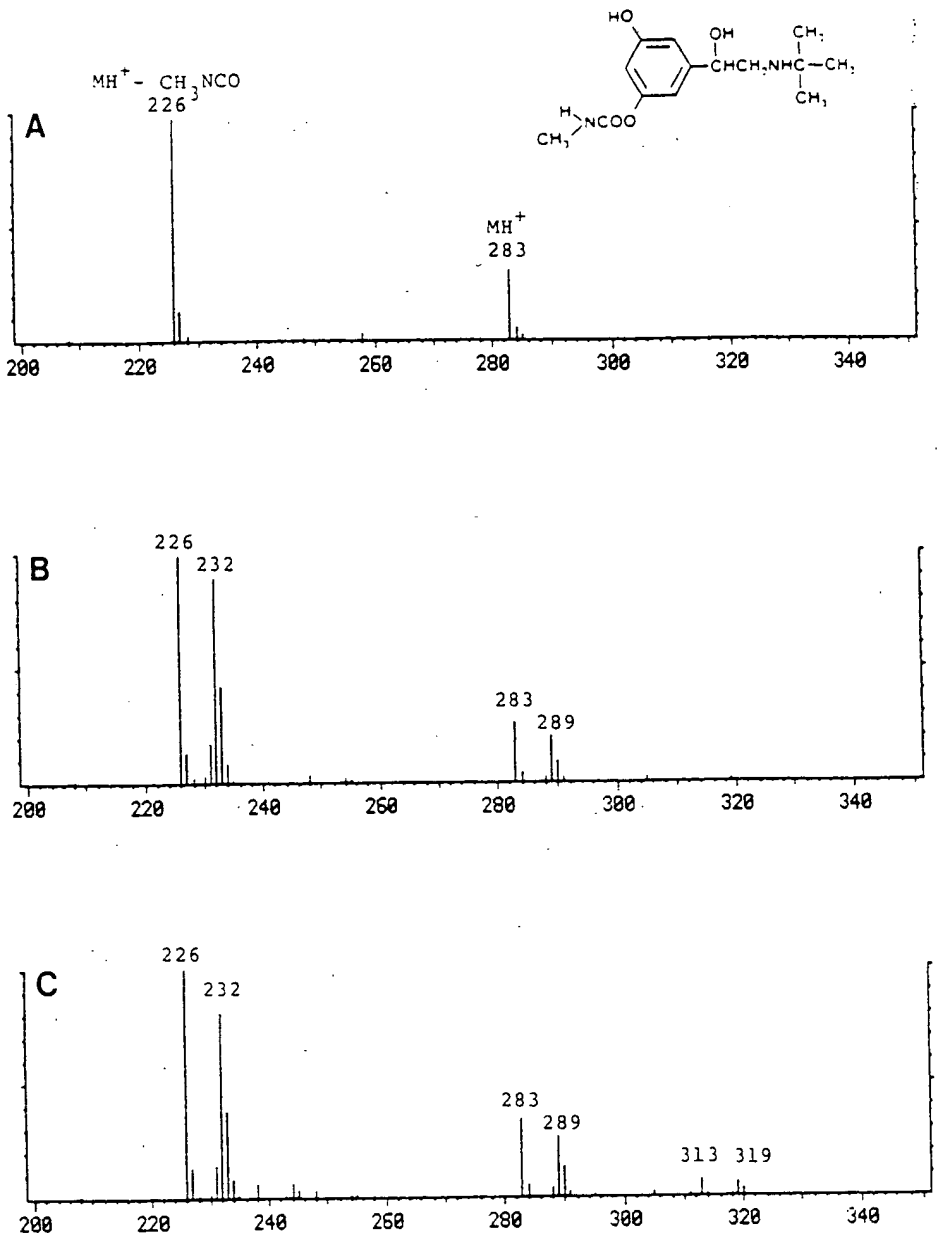

FIG. 9. *TSP mass spectra of compounds related to metabolite fraction V (cf. Fig. 8).*

A, authentic D2468; *B*, compound with R_T = 11.0 min (demethylated monocarbamate); *C*, compound with R_T = 15.6 min (hydroxylated monocarbamate).

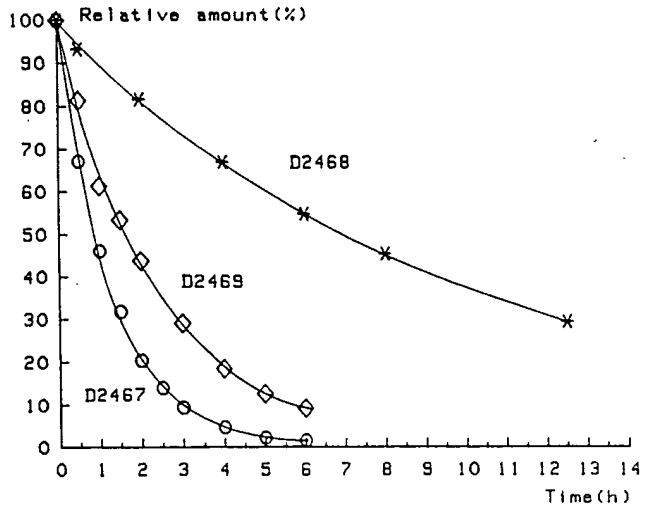

FIG. 10. *Degradation of the N-monomethylcarbamates D2467, D2468, and D2469 during incubation at 37°C in phosphate buffer, pH 7.5.*

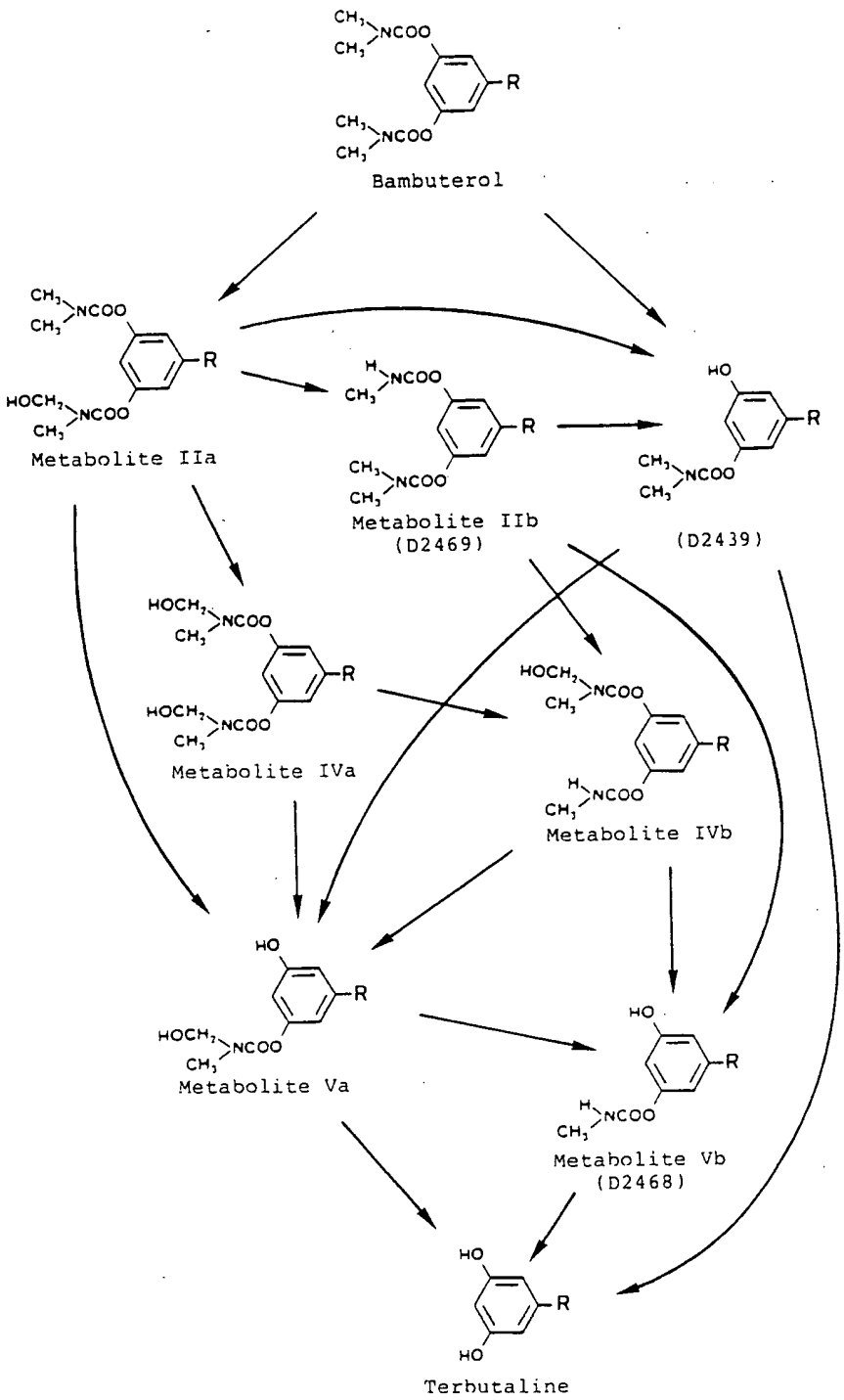

FIG. 11. *Proposed metabolic pathways for the formation of terbutaline from bambuterol.*

$R = CH(OH)CH_2NHC(CH_3)_3$

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0006-2

HYDROLYSIS OF ^{3}H-BAMBUTEROL, A CARBAMATE PRODRUG OF TERBUTALINE, IN BLOOD FROM HUMANS AND LABORATORY ANIMALS *IN VITRO*

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Abstract—Tritiated bambuterol, a bis-dimethylcarbamate prodrug of terbutaline, was incubated *in vitro* with blood from both sexes of the following species: man, guinea pig, rat, mouse, dog and rabbit. The rates of hydrolysis of bambuterol to its monocarbamate derivative and further to terbutaline were measured. Large species variations were observed, e.g. blood from two of the human subjects was 15-fold more active than blood from the male rats. The rate of terbutaline formation as a function of initial bambuterol concentration was investigated in human plasma, and was found to describe a bell-shaped curve.

Several pieces of evidence indicated that butyrylcholinesterase (EC 3.1.1.8) is the blood enzyme predominantly responsible for hydrolysis of bambuterol, although minor contributions from other esterases cannot be excluded. An exception may be blood from the rabbit, where the kinetics of the hydrolysis was different than in blood from the other species.

The kinetics of bambuterol hydrolysis is discussed on basis of the established mechanism of carbamate interactions with cholinesterases, and the high affinity of bambuterol for butyrylcholinesterase.

Bambuterol (1-3,5-bis-(*N*,*N*-dimethylcarbamoyloxy)phenyl)-2-*t*-butylaminoethanol hydrochloride) is a carbamate prodrug of the adrenoreceptor agonist terbutaline (Fig. 1) [1]. The dimethylcarbamate group was used to obtain built-in cholinesterase inhibitory properties in the prodrug in order to improve its presystemic hydrolytic stability. Clinical trials have demonstrated prolonged duration of action and reduced side effects, and pharmacokinetic studies have shown lower peak-trough ratios for plasma terbutaline concentrations, after intake of bambuterol as compared to plain terbutaline [2].

The bioconversion of bambuterol to terbutaline involves, in the simplest case, a two-step hydrolysis with the monocarbamate derivative as intermediate (Fig. 1). Since bambuterol is a carbamate derivative, inhibition of the cholinesterase will occur simultaneously with hydrolysis. It is believed that inhibition is caused by rapid carbamylation of a serine residue at the esteratic active site to generate an inactive carbamylated esterase intermediate which is only slowly hydrolyzed back to active esterase, i.e. k_3 in equation 1 is rate determining. Thus, the "inhibition" is a result of a slow turnover of the catalytic cycle.

$$E + CM \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E - CM \underset{M}{\overset{k_2}{\rightarrow}} EC \xrightarrow{k_3} E + C \qquad (1)$$

where E = esterase, CM = carbamate substrate, M = phenolic product of hydrolysis, EC = carbamylated esterase and C = carbamic acid derivative.

We recently demonstrated that bambuterol has an extremely high affinity for BuChE† (EC 3.1.1.8) in human blood, reflected by an IC_{50} of 17 nM [3]. The present *in vitro* study was designed to investigate bambuterol as a substrate for hydrolytic enzymes in blood from various species. Knowledge of the type obtained in this study is important for the interpretation and understanding of pharmacological effects of bambuterol obtained *in vivo*. In addition, we have investigated in more detail the kinetics of bambuterol hydrolysis in human plasma, with particular emphasis on the effect of the initial bambuterol concentration on the rate of terbutaline formation.

MATERIALS AND METHODS

Animals

Sprague–Dawley rats and NMRI mice, weighing around 250 and 25 g, respectively, were obtained from Möllegaard (Copenhagen, Denmark), Dunkin Hartley guinea pigs weighing 400–500 g from Sahlin (Malmö, Sweden), New Zealand White rabbits weighing around 2 kg from S. Hansson (Dörröd, Sweden), and Beagle dogs, 15–16 months old, from (Turbo-hundar, Sweden). The human subjects were healthy volunteers, 27–34 years of age.

Blood samples

Blood was collected into heparinized Vacutainer tubes. The mouse blood was collected from eight animals of each sex, rat and guinea pig blood from two animals of each sex, and the blood from each sex pooled. Of the other species blood from one single individual of each sex was used for each incubation. The incubations and enzyme activity determinations were started at the most 2 hr after

* Subsidiary of AB Astra.

† Abbreviations used: LC, liquid chromatography; BuChE, butyrylcholinesterase (plasma cholinesterase, acylcholine acylhydrolase, EC 3.1.1.8); IC_{50}, concentration resulting in 50% inhibition of enzyme activity.

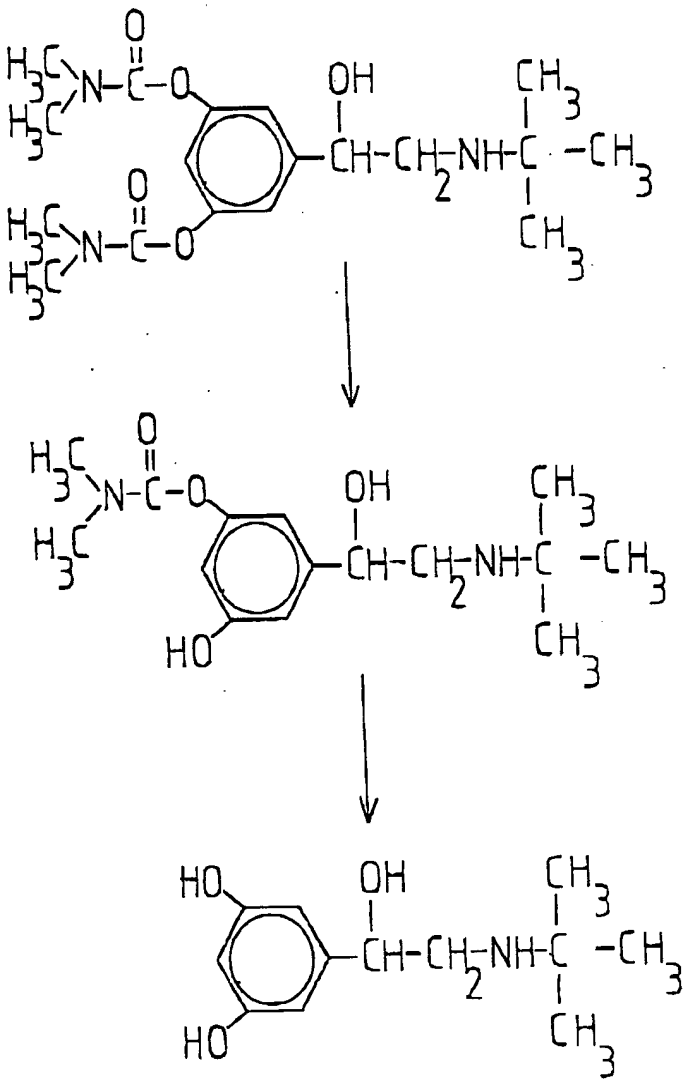

Fig. 1. Two-step hydrolysis of bambuterol to terbutaline.

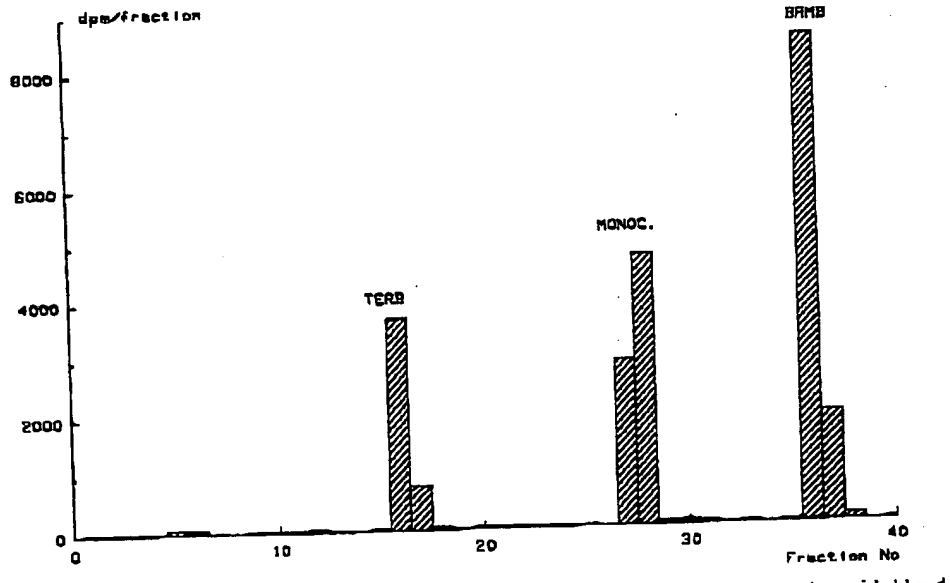

Fig. 2. A typical radiochromatogram. This particular tracing was obtained after incubation with blood from rabbit 1 for 120 min.

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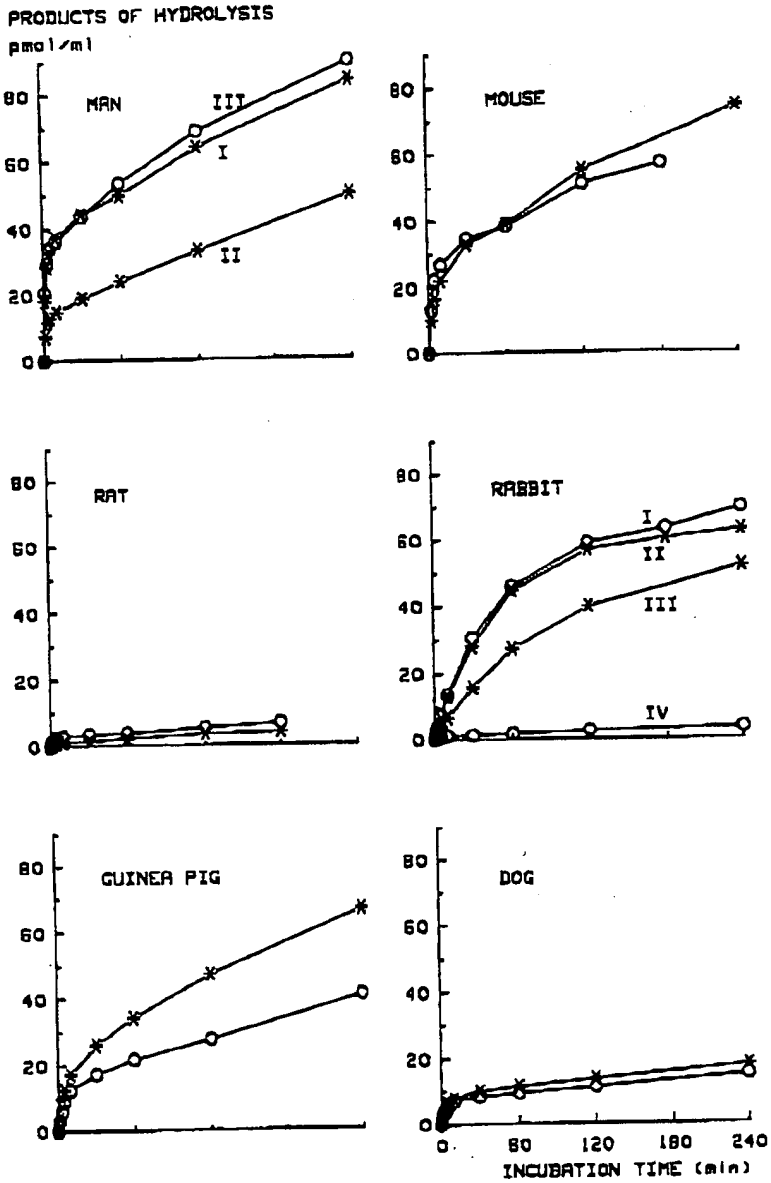


Fig. 3. Hydrolysis of bambuterol in blood from six species. Crosses are males and circles females. Products of hydrolysis are the monocarbamate + terbutaline.

collection of blood. Blood from three humans was tested, and these are referred to as I, II (males) and III (female).

Human blood plasma

Blood from one individual (man I) was collected into vacutainer tubes. Plasma was prepared by centrifugation of the blood for 10 min at 3700 rpm.

Compounds

Tritiated bambuterol hydrochloride (^{3}H-bambuterol, batch TRQ 2750) with a specific activity of 1.02 Ci/mmol was obtained from the Radiochemical Centre (Amersham, U.K.). The label was at the benzylic carbon in the ethanolamine side chain. The radiochemical purity was found to be 96% when the substance was tested on the LC-gradient system described below. One peak of impurity, containing about 1% of the radioactivity, comigrated with the monocarbamate. The stock solution of ^{3}H-bambuterol was 8.4 mM in ethanol. Prior to incubations this stock solution was appropriately diluted with physiological saline. During the incubations performed no tritiated water was detected, thus little or no tritium exchange occurred.

Other chemicals were of analytical grade and purchased from commercial sources.

Preparation of ^{3}H-monocarbamate

The tritiated monocarbamate was prepared by incubating 1 μM ^{3}H-bambuterol with 10 ml freshly prepared human blood plasma for 7 hr at 37° and then for 13 hr at room temperature. Bambuterol and its metabolites were then extracted on a SepPak C_{18}-cartridge (Waters Associates). The metabolite eluate was evaporated to dryness, dissolved in 300 μl of

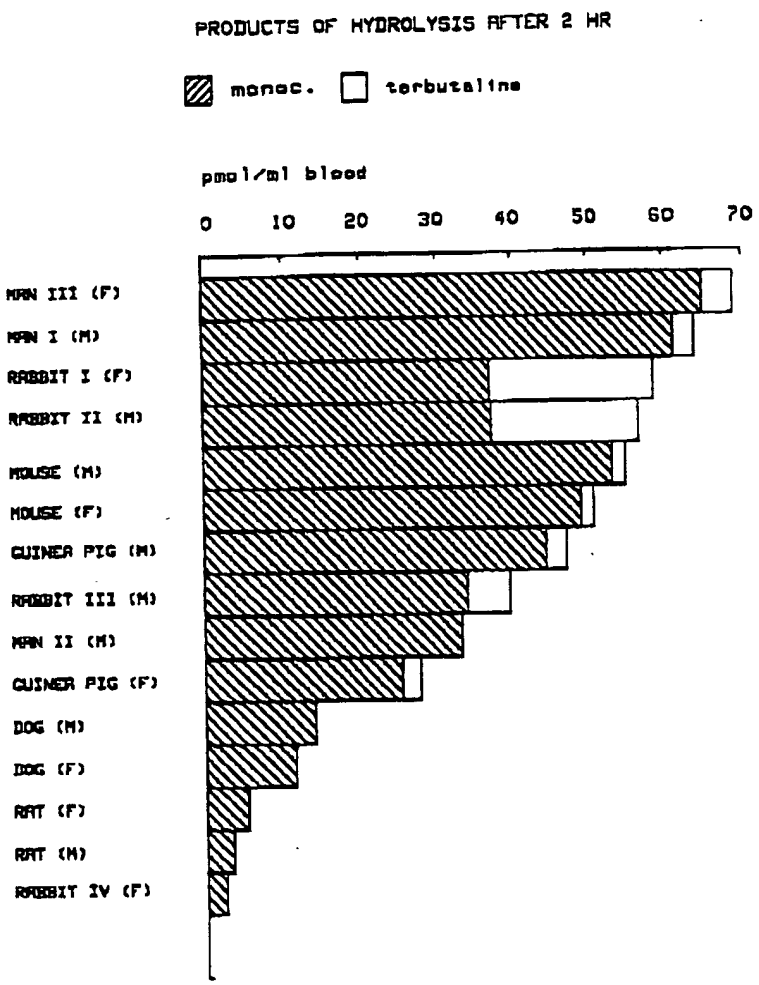


Fig. 4. Products of hydrolysis of bambuterol in blood after 2 hr at 37°. Open parts of the bars are terbutaline and hatched parts of the bars are the monocarbamate.

water and metabolites separated on the LC-system. The fraction with a retention time resembling that of synthetic unlabelled monocarbamate was collected, evaporated to dryness, and dissolved in water.

Incubations

Whole blood. Six millilitres of the blood was temperated at 37° for about 10 min on a shaking water bath. Then the reaction was initiated by adding 300 μl of a 2 μM ^{3}H-bambuterol solution, thus yielding a bambuterol concentration of around 95 nM. After incubation times indicated in the figures aliquots of 500 μl were added to 500 μl 5% perchloric acid. After mixing on a vortex mixer the samples were centrifuged. An aliqout of 200 μl of the supernatant was then injected onto the liquid chromatography system described below.

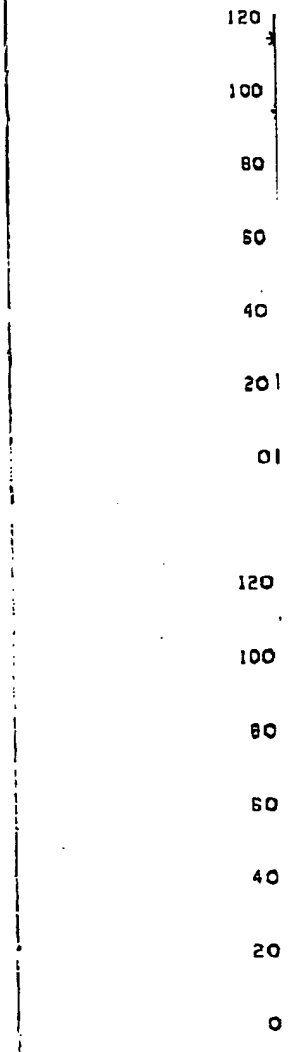
When the inhibition of bambuterol hydrolysis by physostigmine was tested, the blood from man III was preincubated at 37° with 10^{-6} M physostigmine for 15 min before addition of the bambuterol. The physostigmine was added dissolved in 100 μl saline. In a control incubation a blood specimen was preincubated with 100 μl physiological saline.

Except for the mouse, man III and rabbits I and II, where only single incubations were performed, the incubations were run in duplicates. The results given are averages between the duplicate experiments.

Human plasma. These incubations were performed with 5 ml of plasma, to which 250 μl of ^{3}H-bambuterol or ^{3}H-monocarbamate was added. The samples were processed as described above.

Liquid chromatography (LC) system

The LC system consisted of two Waters M-45 pumps, a Waters Intelligent Sample Processor 710 B, and a Waters Automated Gradient controller Model 680. The column was Nucleosil C_{18}, 5 μm (150 × 4-6 m i.d.). Mobile phase A consisted of 3.86 g of ammonium acetate and 3.5 ml of acetic acid diluted with 1000 ml water. Mobile phase B was 3.86 g of ammonium acetate and 3.5 ml of acetic acid diluted with 1000 ml 90% methanol in water (v/v). The flow rate was 1.0 ml/min. The gradient program was as follows: Initial conditions 80% A/20% B, linear change to 50% A/50% B during the first 10 min; linear change to 10% A/90% B during 10–20 min; linear change to 80% A/20% B during 23–26 min. At least 7 min of equilibration time at initial condition was used between the runs.



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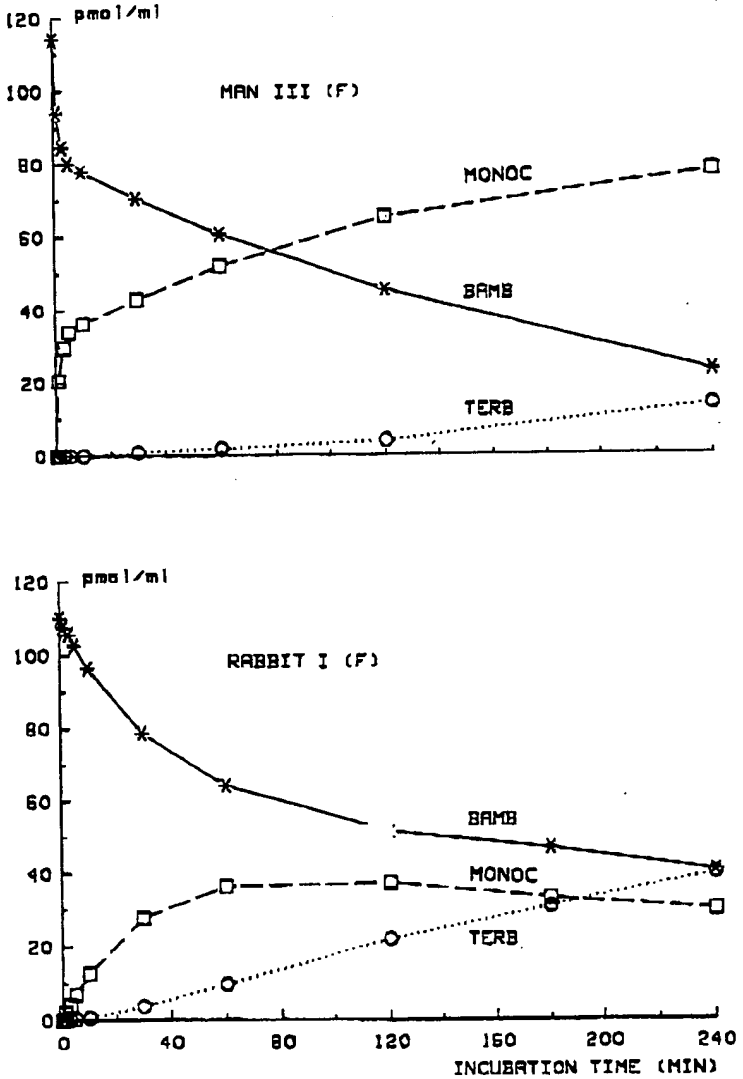


Fig. 5. Formation of the monocarbamate and terbutaline from bambuterol in blood from man III and rabbit I *in vitro*.

Fractionation and quantitation of bambuterol and its metabolites

Bambuterol and its metabolites were quantitated by collecting 0.5-min fraction of the LC-eluate with a LKB Redirac 2112 fraction collector. To each fraction 10 ml Optifluor (Packard) was added, and the radioactivity of the samples counted in a Packard Tri Carb Scintillation Spectrometer. Radiochromatograms such as the one shown in Fig. 2 were obtained. The amount of each compound was then estimated by the sum of the radioactivity of three fractions.

Recovery study

^{3}H-bambuterol (1 μM) was incubated with human whole blood (1 ml) for 6 hr at 37°. The incubations (N = 3) were terminated as described above, and the total amount of radioactivity, and the metabolite composition, of the supernatant was determined. The pellets were then suspended in 2.5% perchloric acid by shaking and sonication. After centrifugation the supernatant was analysed as described above. This procedure was repeated three times.

Determination of BuChE activity

BuChE activity was determined as described in detail elsewhere [3]. The substrate used was butyrylthiocholine. The optimal substrate concentration was found to be 1 mM except for the rat where it was 4 mM. The blood specimens were hemolyzed by 4–20-fold dilution with deionized water, and 100 μl of the hemolyzed blood added to the cuvettes (total volume 3.0 ml).

RESULTS

Recovery studies

The recovery of radioactivity as ^{3}H-bambuterol + ^{3}H-monocarbamate + ^{3}H-terbutaline in the supernatant after precipitation with perchloric acid was in blood 83.6 ± 4·2% (N = 222) and in plasma 93.5 ± 4·4% (N = 34). The recovery study

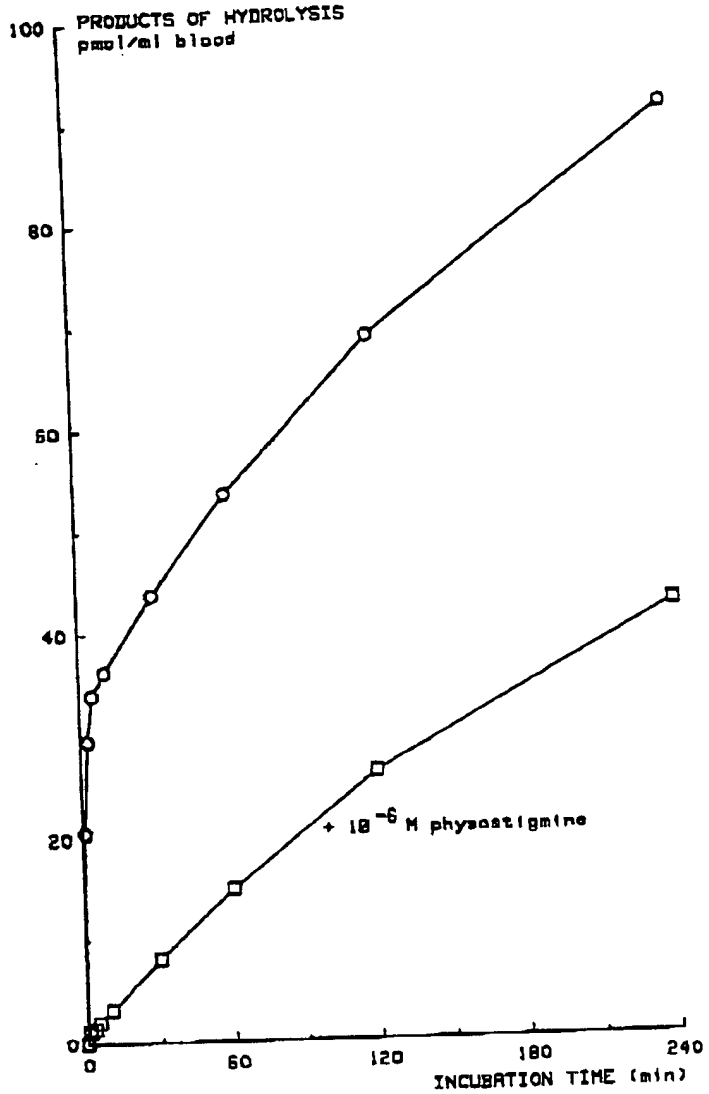


Fig. 6. Inhibition by physostigmine of bambuterol hydrolysis in human blood (man III).

demonstrated that no particular metabolite accumulated in the pellet. Thus, the overall recovery in each LC-run was used for each of these compounds, and all values given have been compensated accordingly.

Hydrolysis of bambuterol in blood from various species

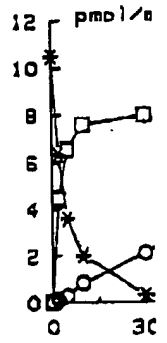
The hydrolysis of approximately 95 nM ^{3}H-bambuterol in blood from males and females of six species is shown in Figs 3 and 4. The blood from the various species had dramatically different capabilities to hydrolyze bambuterol, e.g. two of the human blood specimens were around 30-fold more active in this respect than was blood from one of the female rabbits and 15-fold more active than blood from the male rat.

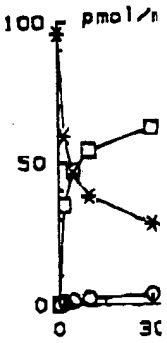
The hydrolysis of bambuterol was non-linear with time as is seen in Fig. 3. The probable biochemical mechanisms are discussed below.

The first product of hydrolysis of bambuterol is the monocarbamate derivative (cf. Fig. 1) and, in five of the species, under the conditions chosen, only a small fraction of this compound was further transformed to terbutaline as illustrated in Fig. 4. The exception was blood from the rabbit. The rates of hydrolysis of bambuterol and the fate of the monocarbamate and terbutaline in blood from man III and rabbit I are compared in Fig. 5. The initial rate of bambuterol hydrolysis and monocarbamate formation was much faster in human than in rabbit blood. However, as is also seen in Fig. 5, the hydrolysis of the monocarbamate to yield terbutaline took place to a much larger extent in rabbit blood.

Correlation between BuChE activity and bambuterol hydrolysis

BuChE activity, with butyrylthiocholine as substrate, was measured in aliquots of the blood specimens from all species used in the bambuterol hydrolysis experiments. There was a good correlation ($e = 0.82$) between the rapid initial phase (0–5 min) of bambuterol hydrolysis and BuChE activity. BuChE activities in blood from man I, II and III





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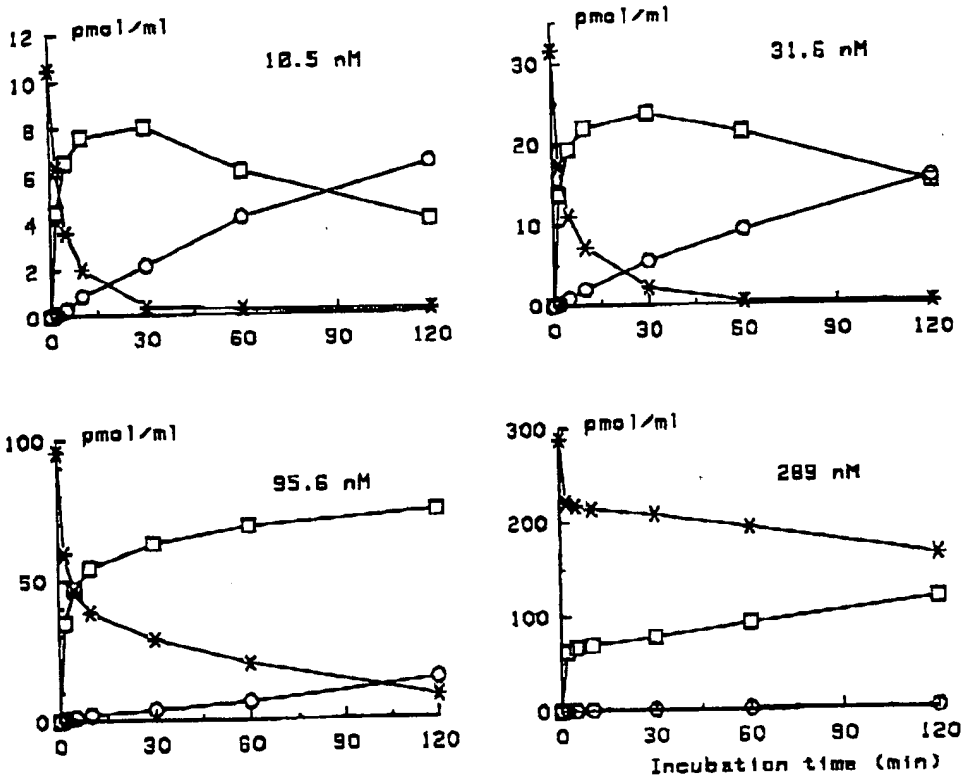


Fig. 7. Hydrolysis of ^{3}H-bambuterol in human plasma at four different bambuterol concentrations. *, bambuterol; □, the monocarbamate, ○, terbutaline.

(Figs 3 and 4) were 14.3, 6.8 and 14.0 nmol thiocholine/min/5 μl blood, respectively.

The effect of physostigmine on the hydrolysis of bambuterol in human blood

The effect of preincubation of blood from man III with 10^{-6} M of the potent cholinesterase inhibitor physostigmine is shown in Fig. 6. The rapid initial phase of bambuterol hydrolysis was completely abolished by physostigmine, while the slow phase was only mildly affected.

The effect of bambuterol concentration on monocarbamate and terbutaline formation in human plasma

The hydrolysis of ^{3}H-bambuterol in human plasma, at four different bambuterol concentrations, is illustrated in Fig. 7. It is very clear, particularly at higher concentrations, that the hydrolysis of bambuterol was a non-linear reaction. The possible biochemical mechanisms underlying this behaviour are discussed below. The capacity of the rapid initial phase was around 60 pmol/ml plasma, a value which is in good agreement with reported concentrations of BuChE in human plasma [4]. At the highest bambuterol concentration this phase was completed within 2 min. The slow phase of hydrolysis proceeded at a rate of around 25 pmol/ml plasma/hr.

The rate of terbutaline formation at the four bambuterol concentrations are compared in Fig. 8. Terbutaline was produced at the highest rate from 31.6 nM bambuterol (9 pmoles terbutaline/ml plasma/hr), while formation was very slow at the highest bambuterol concentration (289 nM).

The rates of hydrolysis of ^{3}H-bambuterol and ^{3}H-monocarbamate were compared as illustrated in Fig. 9. The initial burst so typical for bambuterol hydrolysis was much less pronounced for monocarbamate hydrolysis.

DISCUSSION

Esterases are a heterogeneous group of enzymes, known to differ greatly qualitatively and quantitatively between species [5, 6]. This is true also for BuChE [7]. In humans several genotypes of the enzyme exist, and rare but healthy individuals apparently completely lack this enzyme activity [8, 9]. As indicated in this study, BuChE is probably the enzyme in blood responsible for hydrolysis of bambuterol. Therefore, it was not too surprising to observe the large difference in the rate of bambuterol hydrolysis between blood from the various species (cf. Figs 3 and 4).

The product of hydrolysis of bambuterol is the monocarbamate derivative which may be further hydrolyzed to terbutaline (cf. Fig. 1). It was recently shown that bambuterol has a higher affinity for human BuChE than the monocarbamate [3], since the monocarbamate was found to be a 10-fold less efficient inhibitor of this enzyme. This difference in affinity to BuChE is also reflected by the results presented in Fig. 9. The hydrolysis of the monocarbamate almost completely lacked the initial burst

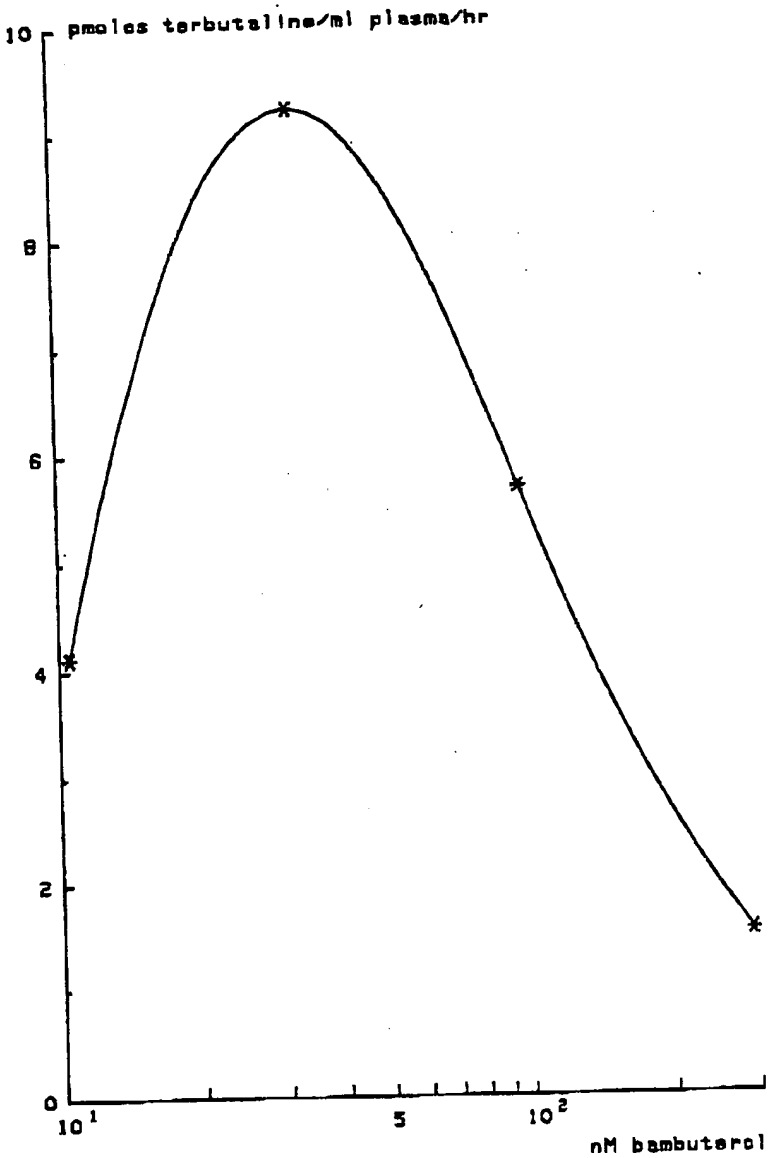
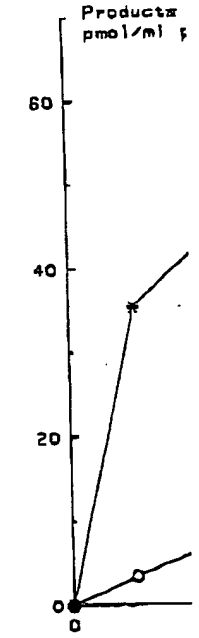


Fig. 8. The formation in human plasma of terbutaline from ^{3}H-bambuterol. These results are calculated from Fig. 7.

so typical for hydrolysis of bambuterol. This should further imply that as long as bambuterol is present in appreciable amounts, the first step of hydrolysis will dominate, and little terbutaline will be formed. The results presented in this study are in accordance with these expectations as illustrated in Figs 5 and 7. In blood from rabbit the situation was different as seen in Fig. 5. Here, the monocarbamate derivative appeared to be a better substrate than bambuterol for the esterases.

In this and a recent report [3] several pieces of evidence (listed below) strongly indicate that BuChE is the most important blood enzyme involved in the hydrolysis of bambuterol, the possible exception is the rabbit. The evidence for a crucial role of BuChE in the hydrolysis of bambuterol in human blood (and plasma) is numerous. Firstly, the capacity of the inital burst of hydrolysis was in the order of 60 pmol/ml (Fig. 7), which corresponds very well with reported values for the concentration of BuChE active sites in human blood plasma [4]. The rate of hydrolysis in the slow phase (Fig. 7) also allows us to calculate the half-time for regeneration of the BuChE activity at hydrolysis of bambuterol. Thus, if the total concentration of enzyme is assumed to be 60 pmol/ml and the rate of hydrolysis in the slow phase was 25 pmol/ml plasma/hr, $t_{1/2}$ will be 72 min for the reactivation of BuChE. This is in excellent agreement with results calculated from inhibition kinetics [3]. This kinetic behaviour of hydrolysis and inhibition is in accordance with the extensively investigated interaction between cholinesterases and carbamates [10, 11]. Thus, k_1 and k_2 in equation 1 control the rapid binding and hydrolysis steps, and k_3 the slow regeneration of the active site. Secondly, there was a good correlation ($r = 0{\cdot}82$) between the rate of bambuterol hydrolysis during the initial phase and the rate of hydrolysis of the BuChE substrate



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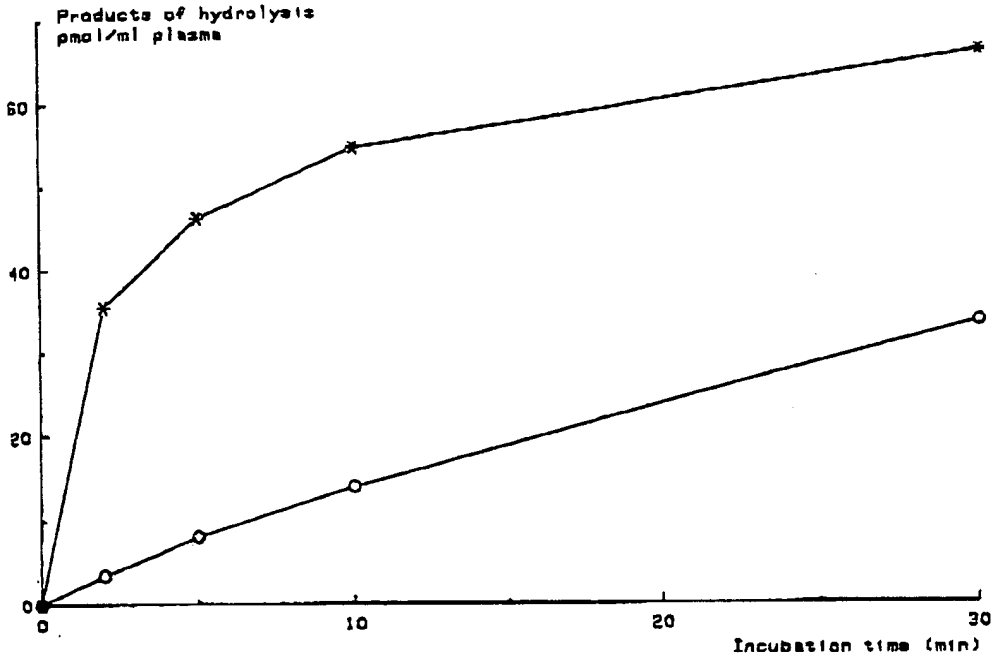

Fig. 9. Hydrolysis of bambuterol and of the monocarbamate in human blood plasma. The substrate concentrations were 100 nM. Results shown represent monocarbamate formed from bambuterol (•), and terbutaline formed from the monocarbamate (○).

butyrylthiocholine. Thirdly, carbamylation of the esterase by preincubation of human blood with physostigmine, a well known reversible cholinesterase inhibitor [10, 11], completely blocked the rapid initial phase of bambuterol hydrolysis (Fig. 6). Bambuterol, however, is a better inhibitor than physostigmine of BuChE and thus probably has a higher affinity to the enzyme's active site [3], so once the *N*-methylcarbamate residue from physostigmine has left the enzyme, bambuterol successfully competed for binding to the active site of the regenerated esterase. Consequently, the slow phase of bambuterol hydrolysis was only mildly affected by physostigmine. The crucial role of BuChE in both the rapid and the slow phase of bambuterol hydrolysis is further supported by experiments in progress in our laboratory using plasma from humans homozygous for the atypical form of BuChE (E_aE_a, kindly provided by Dr J Viby Mogensen, Copenhagen). Both phases of bambuterol hydrolysis are much slower in such atypical plasma.

The kinetics of terbutaline formation from bambuterol has the implication that the amount of terbutaline formed in plasma does not increase with the concentration of bambuterol (Fig. 8). Instead, a level must exist where the rates of inhibition and regeneration of the esterase balance each other to give an optimal rate of terbutaline formation. As can be seen from Fig. 8, of the four bambuterol concentrations used in this study, 31.6 mM resulted in the highest rate of terbutaline formation, while lower and higher concentrations resulted in slower terbutaline formation.

The present study gives insight into the kinetics of hydrolysis in blood and plasma of bambuterol to the monocarbamate derivative and further to the pharmacologically active terbutaline. The blood is, however, only one of many tissues active in biotransformation of bambuterol as will be demonstrated in future reports. It must therefore be remembered, that the dramatic differences in the rate of bambuterol hydrolysis in blood observed in this study will not reflect differences between various animals or individuals in their total capacity of forming terbutaline from bambuterol. Nevertheless, the features of the interaction between bambuterol and BuChE probably positively influence the biological stability of this prodrug. These factors most likely contribute to the sustained generation of the active bronchodilator, terbutaline, resulting in a long duration of action allowing bambuterol to be dosed only once daily in man [12].

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at this temperature for 20 h. (The reaction progress was followed by taking from the mixture a sample which was treated with 2 M sodium hydroxide and extracted with diethyl ether. The extract was subjected to silica TLC with methylene chloride and methanol (10:1).) The reaction mixture was allowed to cool to 120 °C and 2 M sodium hydroxide (650 mL) was added. The temperature was lowered to room temperature and the mixture stirred about 0.5–1 h and extracted with ether. After evaporation, the residue was distilled at 225 °C/1 Torr. Compound 2b (5 g, 37%) was obtained by recrystallization from ligroin (100–140 °C), mp 186–187 °C (lit.[29] mp 186 °C).

9-[(2-Methoxyphenyl)amino]-1,2,3,4-tetrahydroacridine (2c). Phosphorus pentoxide (1.8 mol, 256 g) was mixed with triethylamine hydrochloride (1.8 mol, 448 g) in a flask fitted with a mechanical stirrer and a reflux condenser with a drying tube (calcium chloride) at room temperature. 2-Methoxyaniline (1.8 mol, 222 g) was added dropwise while the mixture was heated in an oil bath to 60 °C (oil-bath temperature). The mixture was further heated to 160 °C until a homogeneous mixture was achieved (0.5 h). The oil-bath temperature was then lowered to 130 °C. Methyl anthranilate (0.3 mol, 45.4 g) and cyclohexanone (0.54 mol, 53.0 g) were added dropwise. The temperature was again increased to 160 °C and maintained at that temperature for 15 min. The reaction mixture was allowed to cool to 110 °C and 2 M sodium hydroxide (4000 mL) was added. The aqueous suspension was extracted with 2 × 1500 mL of diethyl ether. The solvent was stripped off under reduced pressure. The residue was further evaporated at 0.1 mmHg to remove 2-methoxyaniline. A black solid formed and was recrystallized twice from ligroin (80–100 °C) to yield 2c (32.4 g, 36%) as white-yellow crystals: mp 131–132 °C; ^{1}H NMR ($CDCl_3$/TMS) δ/ppm 1.91 (m, 4 H), 2.79 (m, 2 H), 3.19 (m, 2 H), 4.02 (s, 3 H), 6.27 (s, NH), 6.10–8.20 (m, 8 H); ^{13}C NMR ($CDCl_3$/TMS) δ/ppm 25.09 (C-1), 22.75 (C-2), 22.49 (C-3), 33.98 (C-4), 159.77 (C-4a), 128.60 (C-5) 128.41 (C-6), 124.84 (C-7), 123.08 (C-8), 123.67 (C-8a), 142.96 (C-9), 124.45 (C-9a), 147.24 (C-10a); MS *m/e* 304 (100), 289 (13), 273 (11), 197 (11), 182 (11). Anal. ($C_{20}H_{20}N_2O$) C, H, N.

9-[(4-Nitro-2-methoxyphenyl)amino]-1,2,3,4-tetrahydroacridine (3). Compound 2c (20 mmol, 6.1 g) was dissolved in acetic acid (99%, 80 mL). After addition of nitric acid (68%) at 70 °C and stirring for 4 h, it was poured into ice (500 mL). When the ice had melted, 2 M sodium hydroxide (0.5 L) was added. A yellow precipitate 6.5 g (ortho and paraisomer) was washed with water and dried in vacuum. Recrystallization twice from ethyl acetate yielded 3 (2.4 g, 34%), mp 221–223 °C. Anal. ($C_{20}H_{19}N_3O_3$) C, H, N.

9-[(4-Amino-2-methoxyphenyl)amino]-1,2,3,4-tetrahydroacridine (4). A mixture of 3 (13 mmol, 4.6 g), tin (83 mmol, 9.9 g), and 6 M hydrochloride acid (100 mL) was heated with stirring to 120 °C (oil-bath temperature) by which time the tetrahydroacridine had dissolved. The mixture was refluxed for 2 h and cooled. The precipitate was dissolved in water and, after addition of 2 M sodium hydroxide (50 mL), extracted into chloroform. The chloroform extract was evaporated and the residue was recrystallized from 50% ethanol to yield 4 (2.5 g, 56%), mp 155–156 °C.

***N*-[4-[(1,2,3,4-Tetrahydro-9-acridinyl)amino]-3-methoxyphenyl]methanesulfonamide (2d).** Compound 4 (7.5 mmol, 2.4 g) was dissolved in 25 mL of dry pyridine. Mesyl chloride (15 mmol, 1.16 mL) was added slowly at −5 °C and the mixture was stirred for 1 h. The mixture was evaporated under reduced pressure and dissolved in water (200 mL). Hydrochloric acid (4 M, 20 mL) was added to precipitate the hydrochloride, which was taken up in 200 mL of water. 10 mL of sodium hydrogen carbonate was added and the precipitate was crystallized from ethanol (96%) to yield 2d (1.5 g, 50%), mp 248–249 °C (lit.[30] mp 243–245 °C.

NMR Experiments. All DNA samples for NMR were made 5 mM in DNA phosphate by diluting with distilled water. This gave $[Na^+]/[P]$ ratios of 1.2. The NMR intercalator titrations were performed by adding successive aliquots (corresponding to $r \sim 0.005$) of a drug stock solution directly to the DNA solution in the NMR tube. For titrations up to $r \sim 0.05$, the volume increases by 10%. A control experiment, in which water alone was added up to 20% volume increase, showed that T_1, within measuring uncertainties, was unchanged by such dilution. pH was measured to be 7 both before and after the addition of intercalators.

^{23}Na NMR spectra were recorded at 5.9 T on a Bruker AC 250 and obtained without lock. The inversion-recovery (180°–τ–90°–acq) pulse sequence was used for the T_1 measurements with 15 different values of τ for each experiment. The T_1 values were obtained by a three-parameter linear least square fitting procedure. Each T_1 value is the average of at least two measurements. The temperature for the NMR measurements was 27 °C.

^{13}C and ^{1}H spectral data given were also obtained at 5.9 T on a Bruker AC 250 NMR instrument.

Registry No. 1, 321-64-2; 2a, 14807-16-0; 2b, 110245-49-3; 2c, 123333-18-6; 2d, 111232-55-4; 3, 123333-19-7; 4, 123333-20-0; *o*-$NH_2C_6H_4CO_2Me$, 134-20-3; 4-methylcyclohexanone, 589-92-4; cyclohexanone, 108-94-1; aniline, 62-53-3; 2-methoxyaniline, 90-04-0.

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Cyclization-Activated Prodrugs. Basic Carbamates of 4-Hydroxyanisole

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Merck Sharp & Dohme Research Laboratories, West Point, Pennsylvania 19486. *Received January 18, 1989*

A series of basic carbamates of 4-hydroxyanisole was prepared and evaluated as progenitors of this melanocytotoxic phenol. All of the carbamates were relatively stable at low pH but released 4-hydroxyanisole cleanly at pH 7.4 at rates that were structure dependent. A detailed study of the *N*-methyl-*N*-[2-(methylamino)ethyl]carbamate showed that generation of the parent phenol followed first-order kinetics with $t_{1/2}$ = 36.3 min at pH 7.4, 37 °C, and was accompanied by formation of *N,N'*-dimethylimidazolidinone. These basic carbamates are examples of cyclization-activated prodrugs in which generation of the active drug is not linked to enzymatic cleavage but rather depends solely upon a predictable, intramolecular cyclization–elimination reaction.

Esterification of therapeutically active agents to provide prodrugs with improved properties has become a familiar strategy for the circumvention of adverse physicochemical limitations. Ester prodrugs of alcohols and phenols are frequently explored to improve solubility, absorption, and bioavailability and to extend the duration of action of the parent drug.[1,2] It is of course essential for the success of this strategy that the ester progenitor be capable of delivering the parent drug at a practical rate in vivo. Generally, ester prodrugs have depended upon chemical or

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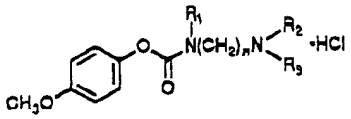
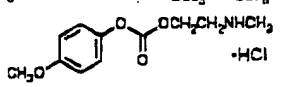
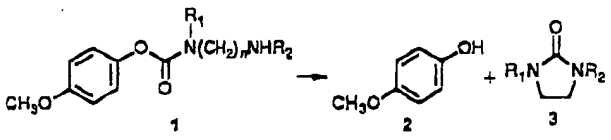
Table I. Carbamates of 4-Hydroxyanisole. Chemical Properties and Half-Lives for Conversion to 4-Hydroxyanisole



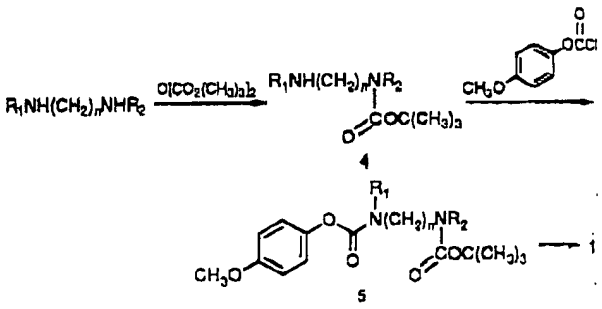
compd[a]	n	R_1	R_2	R_3	mp, °C	$t_{1/2}$, min, at 37 °C pH 7.4	pH 6.8	$t_{1/2}$ at 37 °C pH 5.2	pH 4.2
1a[b]	2	CH_3	CH_3	H	148.0–19.0 (softens at 125)	36.3 ± 1.3 56.0[c] ± 1.4	140 ± 5	57.8 ± 1.9 h	>15 days
1b	2	CH_3	CH_3	CH_3	153.0–154.5	39.6 ± 0.7			
1c	2	C_2H_5	C_2H_5	H	149.0–150.0	118 ± 2			
1d[d]	2	CH_3	H	H	152.5–154.5	304 ± 1			
1e	2	H	CH_3	H	159.0–160.5	335 ± 1			
1f[e]	2	H	H	H	185.0–192.0	724 ± 2			
1g[f]	3	H	H	H	162.0–165.0 (softens at 110)	910 ± 10			
1h	3	CH_3	CH_3	H	143.0–145.0	942 ± 1			
8					53.0–55.0	<0.3			

[a] Anal. C, H, N. [b] pK_a = 8.88. [c] Murine plasma. [d] pK_a = 8.90. [e] pK_a = 8.65. [f] pK_a = 9.60.

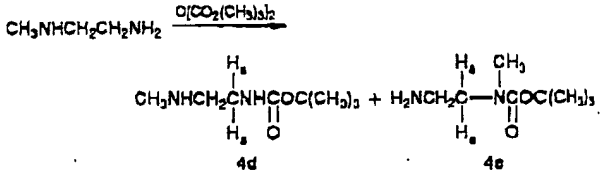
Scheme I



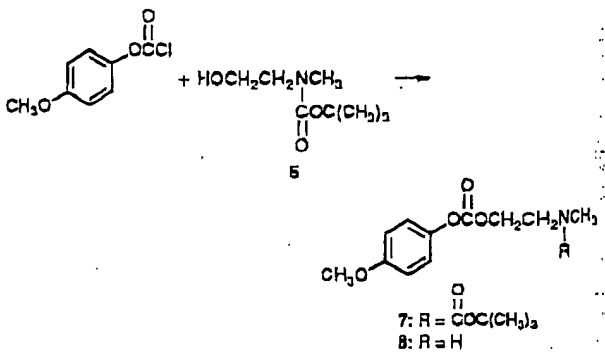
Scheme II



Scheme III



Scheme IV



enzymatic hydrolysis of the ester bond for useful rates of conversion of prodrug to drug. However, this requirement is not always attainable or may be subject to much variability between species or even between individual members of a particular species.

An alternate approach to this problem is release of the parent drug from the prodrug through mechanisms not involving hydrolysis of the ester bond, but rather through an intramolecular cyclization–elimination reaction such as that depicted in Scheme I. In this way, ideally, generation of active drug is not dependent upon the host environment but rather solely upon the rate of the cyclization reaction.

In this work we describe the synthesis of basic carbamates 1 of the clinically effective melanocytotoxic agent 4-hydroxyanisole (2)[3,4] and their evaluation as progenitors of this phenol by an intramolecular cyclization reaction.

Chemistry

Synthesis of the basic carbamates 1 required monoprotected diamine intermediates which could ultimately be deblocked in the last step without destruction of the carbamate function. The *tert*-butoxycarbonyl (BOC) group appeared to be compatible with this approach. Although several different syntheses of mono-alkoxycarbonyl-protected diamines have been reported,[5-10] we found that direct acylation of excess diamine with di-*tert*-butyl dicarbonate ($^1/_8$ molar equiv) in THF (Scheme II) proved to be a convenient source of the protected diamines 4 (Table II). The products from symmetrical diamines were generally of satisfactory purity for use in the next step without requiring extensive purification.

In the case of the unsymmetrical diamine *N*-methylethylenediamine, acylation occurred at each nitrogen to provide 4d and -e in 12 and 38% yields, respectively, after separation by flash chromatography (Scheme III). Structural assignments could be made from the ^{1}H NMR

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Table II. Mono-B

compd	n	R_1
4a	2	CH_3
4c	2	C_2H_5
4d	2	CH_3
4e	2	H
4f	2	H
4g	3	H
4h	3	CH_3

[a] $CDCl_3$. [b] Anal. (

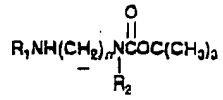
of the correspond
NMR spectrum
protons as a quar
those of 4e were
hydrogen fumarat
peak while that o
The basic carb
reaction of BOC-
phenyl chloroform
group with anhyc
under acidic condi
carbamates as th
trimethyl derivat
from reaction of
4-methoxyphenyl
Basic carbonat
carbamate 1a by
methylamino)eth

Results and Dis

Stability of th
aqueous solution
by HPLC analysi
generated smoot
depending upon t
Detailed kinetic a
1a showed that the
kinetics.
Confirmation t
the cyclization m
the isolation of $N_$
= CH_3) in 49.5%
7.4. In addition, f
at pH 6.8 than at
stable at even lo
nonprotonated an
occur and further
result of a cycliza
The *N*-methyl
formation of 4-h

(11) Zabik, M. J.; S
(12) Adams, P.; Bar
(13) Christenson, I.

Table II. Mono-BOC-Protected Diamines



compd	n	R_1	R_2	yield, %	method	^{1}H NMR, δ (H)	hydrogen fumarate mp, °C dec	hydrogen fumarate ^{1}H NMR, δ (H)
4a	2	CH_3	CH_3	81	B	1.46 (s, 9), 2.48 (s, 3), 2.75 (br t, 2), 2.88 (s, 3), 3.35 (br t, 2)[a]		
4c	2	C_2H_5	C_2H_5	83	B	1.10 (t, t, 6), 1.45 (s, 9), 1.72 (br s, 1), 2.72 (t, q, 4), 3.28 (m, 4)[a]		
4d	2	CH_3	H	12	B		118.0–120.0[b]	1.38 (s, 9), 2.45 (s, 3), 2.80 (t, 2), 3.16 (q, 2), 6.43 (s, 2), 7.17 (m, 1)[c]
4e	2	H	CH_3	38	B		146.0–148.0[b]	1.40 (s, 9), 2.80 (s 3), 2.88 (t, 2), 3.36 (t, 2), 6.44 (s, 2)[c]
4f	2	H	H	94	A	1.38 (s, 9), 2.53 (t, 2), 2.91 (q, 2), 6.72 (br, s, 1)[c]	172.5–173.5[b]	1.38 (s, 9), 2.80 (t, 2), 3.16 (q, 2), 6.44 (s, 2), 7.14 (t, 1)[c]
4g	3	H	H	85	A		147.0–148.0[b]	1.38 (s, 9), 1.65 (m, 2), 2.75 (t, 2), 2.95 (q, 2), 6.43 (s, 2), 6.98 (br t, 1)[c]
4h	3	CH_3	CH_3	72	B	1.48 (s, 9), 1.74 (m, 3), 2.44 (s, 3), 2.58 (t, 2), 2.83 (s, 3), 3.29 (br s, 2)[a]		

[a] $CDCl_3$. [b] Anal. C, H, N. [c] DMSO-d_6.

of the corresponding hydrogen fumarate salts. The ^{1}H NMR spectrum of 4d hydrogen fumarate showed H_a protons as a quartet (coupling with the BOC NH) while those of 4e were found to be a triplet. In addition, the hydrogen fumarate salt of 4e did not exhibit a BOC NH peak while that of 4d did.

The basic carbamates of Table I were synthesized by reaction of BOC-protected diamines 4 with 4-methoxyphenyl chloroformate[11] followed by removal of the BOC group with anhydrous HCl (Scheme II). Deprotection under acidic conditions allowed direct isolation of the basic carbamates as the stable HCl salts in high yields. The trimethyl derivative 1b (Table I) was obtained directly from reaction of *N,N,N'*-trimethylethylenediamine with 4-methoxyphenyl chloroformate.

Basic carbonate 8 was prepared for comparison with carbamate 1a by a similar sequence using (*N*-BOC-*N*-methylamino)ethanol (6) (Scheme IV).

Results and Discussion

Stability of the carbamates of Table I at 37 °C in aqueous solution at pH 7.4 was determined conveniently by HPLC analysis. In every case, 4-hydroxyanisole was generated smoothly and cleanly but at different rates depending upon the structure of the particular carbamate. Detailed kinetic analysis of the *N,N'*-dimethylcarbamate 1a showed that the phenol was formed following first-order kinetics.

Confirmation that 4-hydroxyanisole is formed through the cyclization mechanism of Scheme I was obtained by the isolation of *N,N'*-dimethylimidazolidinone (3, $R_1 = R_2 = CH_3$) in 49.5% yield after heating 1a at 37 °C and pH 7.4. In addition, formation of 4-hydroxyanisole was slower at pH 6.8 than at pH 7.4, and the carbamates are more stable at even lower pH (Table I). This suggests that a nonprotonated amine function is critical for reaction to occur and further substantiates that phenol release is the result of a cyclization reaction.

The *N*-methyl substituent of 1a ($R_1 = CH_3$) precludes formation of 4-hydroxyanisole through an elimination mechanism involving an isocyanate intermediate. In addition, carbamates with N–H substituents normally react faster than *N*-alkyl analogues by this route.[14,15] However, in this series, the N–H derivative 1e formed 4-hydroxyanisole at a much slower rate than the corresponding *N*-methylcarbamate 1a.

Although carbamate esters have been shown to be stable under mild hydrolytic conditions and unreactive toward intermolecular amine addition, reaction with intramolecular nucleophiles may occur with great facility. For example, cyclization of phenyl *N*-(2-aminophenyl)-*N*-methylcarbamate to 1-methyl-2-benzimidazolone proved to be very rapid under conditions where bimolecular reaction of the carbamate with amines could not be detected.[16]

Inspection of Table I shows that the trimethyl analogue 1b generates 4-hydroxyanisole as well as the dimethyl derivative 1a. In this case, addition of the dimethylamino group to the carbamate carbonyl followed by elimination of 4-hydroxyanisole is still possible.[17] All other changes in the carbamate portion of 1a that were examined led to a slower release of the phenol. These included conversion of the N-substituent to ethyl (1c), deletion of *N*-alkyl groups (1d–f), or extension of the carbon chain to three methylenes (1g and –h). The carbonate 8 corresponding to carbamate 1e was found to be extremely reactive at pH 7.4 and 37 °C and released the phenol with $t_{1/2}$ <20 s. This carbonate, like the carbamates, was also relatively stable under more acidic conditions.

The *N,N'*-dimethylcarbamate 1a was also found to be stable toward murine plasma esterases. Formation of 4-hydroxyanisole from 1a was not faster in murine plasma at pH 7.4 and 37 °C ($t_{1/2}$ = 56.0 min) when compared to

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nonenzymatic conditions, but even slightly slower. This apparent stabilizing effect of the murine plasma could be due to binding of the carbamate to plasma proteins, resulting in partial inhibition of the cyclization mechanism. Resistance of carbamates to enzymatic hydrolysis has been noted previously.[20-23]

This series of basic carbamates of 4-hydroxyanisole appears to satisfy the requirements for cyclization-activated prodrugs. That is, formation of the active drug is not linked to enzymatic cleavage but rather depends solely upon a predictable, intramolecular cyclization–elimination reaction. The pH dependence of the critical cyclization step can also be advantageous in the design of practical prodrugs. For example, relatively stable, weakly acidic solutions of the basic carbamate can be prepared prior to administration. Also, the release of a relatively lipophilic and neutral or weakly acidic drug from a basic, hydrophilic prodrug offers interesting possibilities which might be exploited for drug distribution applications.

Experimental Section

All melting points were obtained on a Thomas-Hoover Unimelt capillary melting point apparatus using open capillaries and are uncorrected. Analytical results are indicated by atom symbols and are within ±0.4% of theoretical values. ^{1}H NMR spectra were recorded for all intermediates and final products on either a Varian XL-300 or a GE NT-360 instrument using tetramethylsilane as an internal standard and are consistent with assigned structures. E. Merck silica gel, 230–400 mesh, was used for the flash chromatographies.

Preparation of Mono-BOC Diamines. Method A. *tert*-Butyl *N*-(2-Aminoethyl)carbamate (4f). A solution of di-*tert*-butyl dicarbonate (7.27 g, 33.3 mmol) in THF (30 mL) was added over 30 min to a stirred, cooled solution of ethylenediamine (6.7 mL, 100 mmol) in THF (30 mL) at 0 °C. After addition was complete, the reaction mixture was stirred in an ice bath for 30 min and then at room temperature for 18 h. Solvents were removed under reduced pressure, and the residue was partitioned between EtOAc and brine. The EtOAc extract was washed with brine, dried (Na_2SO_4), filtered, and concentrated to 5.0 g (94%) of clear oil.

Method B. *tert*-Butyl *N*-[2-(Methylamino)ethyl]carbamate (4d) and *tert*-Butyl *N*-Methyl-*N*-(2-aminoethyl)carbamate (4e). A solution of di-*tert*-butyl dicarbonate (3.27 g, 15 mmol) in THF (30 mL) was added over 35 min to a stirred, ice bath cooled solution of *N*-methylethylenediamine (4.4 mL, 50 mmol) in THF (100 mL). After stirring at room temperature overnight, the reaction was processed by the same procedure as in method A. Flash chromatography over silica gel and elution with 20% MeOH–80% $CHCl_3$ gave first 1.0 g (38%) of 4e as the faster moving material and then 0.30 g (12%) of 4d as the slower moving product.

Preparation of 4-Hydroxyanisole Carbamates. General Procedure. *N*-Methyl-*N*-[2-(methylamino)ethyl]carbamic Acid 4-Methoxyphenyl Ester Hydrochloride (1a). A solution of *tert*-butyl *N*-methyl-*N*-[2-(methylamino)ethyl]carbamate (3.0 g, 16.1 mmol) and *N,N*-diisopropylethylamine (2.8 mL, 16.1 mmol) in THF (50 mL) was added over 30 min to a stirred, cooled solution of 4-methoxyphenyl chloroformate[11] (3.0 g, 16.1 mmol) in THF (50 mL). After addition was complete, the reaction mixture was stirred in an ice bath for 30 min and then at room temperature for 20 h. Solvents were removed under reduced pressure, and the residue was partitioned between EtOAc and brine. The EtOAc extract was dried (Na_2SO_4), filtered, and concentrated. Flash chromatography of the residue over silica gel and elution with 15% EtOAc–85% *n*-butyl chloride gave pure BOC-protected carbamate (4.9 g, 90%) as a clear oil.

A solution of the BOC-protected carbamate (4.9 g, 14.5 mmol) was dissolved in EtOAc (100 mL) and cooled in an ice bath. After saturating with anhydrous HCl, the reaction mixture was allowed to warm to room temperature over 3 h. Solvents were removed under reduced pressure, and the residue was recrystallized from MeOH–EtOAc–hexane to give *N*-methyl-*N*-[2-(methylamino)-ethyl]carbamic acid 4-methoxyphenyl ester hydrochloride (3.17 g, 79.6%), mp 148.0–149.0 °C with softening at 125 °C.

***N*-Methyl-*N*-[2-(dimethylamino)ethyl]carbamic Acid 4-Methoxyphenyl Ester Hydrochloride (1b).** A solution of *N,N,N'*-trimethylethylenediamine (1.3 mL, 10 mmol) and *N,N*-diisopropylethylamine (1.75 mL, 10 mmol) in THF (20 mL) was added over 0.5 h to a stirred, cooled solution of 4-methoxyphenyl chloroformate[11] (1.86 g, 10 mmol) in THF (30 mL). After stirring at ice bath temperature for 1 h and then at room temperature for 2 h, solvent was removed under reduced pressure and the residue partitioned between EtOAc and H_2O. The organic extract was washed with brine, dried (Na_2SO_4), filtered, and concentrated. Flash chromatography of the residue over silica gel and elution with 5% MeOH–95% $CHCl_3$ gave 1.0 g (35%) of product as an oil. The HCl salt, mp 153.0–154.5 °C, was prepared with anhydrous HCl in EtOH followed by recrystallization from MeOH–EtOAc.

Isolation of *N,N'*-Dimethylimidazolidinone from Hydrolysis of 1a. A solution of *N*-methyl-*N*-[2-(methylamino)-ethyl]carbamic acid 4-methoxyphenyl ester hydrochloride (1a) (500 mg, 1.82 mmol) in pH 7.4 phosphate buffer (25 mL) was heated at 37 °C for 6.5 h and then cooled to room temperature overnight. The pH of the solution was maintained at 7.3–7.5 by the dropwise addition of 1 N NaOH. After lyophilization, the residue was extracted with CH_2Cl_2, which was then filtered, dried (Na_2SO_4), filtered, and concentrated to 272 mg of liquid. ^{1}H NMR analysis showed that *N,N'*-dimethylimidazolidinone was present (49.5% yield). To further substantiate the presence of the imidazolidinone, this product was dissolved in $CHCl_3$ extracted with 10% NaOH then H_2O to remove 4-methoxyphenol, dried (Na_2SO_4), filtered, and concentrated. The residue was distilled (bath temperature 120 °C, 14 mmHg) to give 30 mg (14.4%) of *N,N'*-dimethylimidazolidinone identical with an authentic sample by ^{1}H NMR and MS.

***tert*-Butyl *N*-(2-Hydroxyethyl)-*N*-methylcarbamate (5).** A solution of di-*tert*-butyl dicarbonate (43.6 g, 0.20 mol) in DMF (100 mL) was added over 45 min to a stirred solution of *N*-methylethanolamine (15.0, 0.20 mol) in DMF (250 mL) at 10–15 °C. After stirring at room temperature overnight, DMF was mol) at 50 °C and 0.1 mmHg, and the residue was dissolved in EtOAc. The EtOAc extract was washed with H_2O, dried (Na_2SO_4), filtered, and concentrated. Distillation gave the BOC-protected alcohol (25.7 g, 73.5%): bp 83–84 °C (0.05 mmHg); ^{1}H NMR ($CDCl_3$) δ 1.47 (s, 9 H), 2.92 (s, 3 H), 3.41 (m, 2 H), 3.77 (m, 2 H).

4-Methoxyphenyl 2-(Methylamino)ethyl Carbonate Hydrochloride (8). 4-Methoxyphenyl chloroformate[11] (0.94 g, 5.4 mmol) was added to a solution of *tert*-butyl *N*-(2-hydroxyethyl)-*N*-methylcarbamate (0.88 g, 4.7 mmol) and the mixture stirred at room temperature overnight. After concentrating under reduced pressure at 45 °C, the residue was partitioned between EtOAc and H_2O containing a little NaOH. The EtOAc was washed with brine, dried (Na_2SO_4), filtered, and concentrated. Flash chromatography over silica gel and elution with $CHCl_3$ gave 1.9 g of the protected carbonate as a clear, colorless oil: ^{1}H NMR ($CDCl_3$) δ 1.48 (s, 9 H), 2.95 (s, 3 H), 3.55 (m, 2 H), 3.80 (s, 3 H), 4.32 (m, 2 H), 6.89 (d, 2 H), 7.10 (d, 2 H).

A solution of this BOC-protected carbonate (1.9 g) in dry EtOAc (30 mL) was cooled in an ice bath and saturated with anhydrous HCl. After stirring at ice bath temperature for 20 min and then at room temperature for 30 min, solvents were removed under reduced pressure and the residue was recrystallized from MeOH–EtOAc–hexane to give the deprotected carbonate 8 (0.75 g, 61%).

Determination of Carbamate Half-Lives in Buffer. Buffer solution (2.0 mL), preheated to 37 °C, was added quickly to approximately 0.5 mg of the carbamate hydrochloride salt to give a final concentration of approximately 1 mM. The resulting solution was heated at 37 °C while 20-μL samples were removed at intervals and injected directly into the HPLC injection port-

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clear oil.
g (4.9 g, 14.5 mmol)
an ice bath. After
mixture was allowed
ents were removed
recrystallized from
(2-(methylamino)-
ydrochloride (3.17
g at 125 °C.
l]carbamic Acid
(1b). A solution of
10 mmol) and *N,N*-
THF (20 mL) was
of 4-methoxyphenyl
mL). After stirring
room temperature
d pressure and the
The organic extract
d, and concentrated.
ilica gel and elution
%) of product as an
prepared with an-
rystallization from

dinone from Hy-
-[2-(methylamino)-
hydrochloride (1a)
buffer (25 mL) was
room temperature
tained at 7.3–7.5 by
lyophilization, the
s then filtered, dried
of liquid. ^{1}H NMR
idinone was present
presence of the im-
HCl_3 extracted with
phenol, dried (Na_2
e was distilled (bath
mg (14.4%) of *N*-
in authentic sample

thylcarbamate (6).
g, 0.20 mol) in DMF
rred solution of *N*-
F (250 mL) at 10–15
light, DMF was mol)
dissolved in EtOAc
ed (Na_2SO_4), filtered,
C-protected alcohol
); ^{1}H NMR ($CDCl_3$)
, 3.77 (m, 2 H).
hyl Carbonate Hy-
ormate[11] (0.94 g, 5.4
utyl *N*-(2-hydroxy-
ol) and the mixture
concentrating under
partitioned between
1. The EtOAc was
l, and concentrated.
ion with $CHCl_3$ gave
olorless oil: ^{1}H NMR
, 2 H), 3.80 (s, 3 H),

(1.9 g) in dry EtOAc
ated with anhydrous
for 20 min and then
were removed under
recrystallized from
ed carbonate 8 (0.75

s in Buffer. Buffer
as added quickly to
ochloride salt to give
mM. The resulting
mples were removed
PLC injection port.

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In some cases, 0.10-mL aliquots were quenched in 1 N HCl (1.8 mL) prior to injection. Unreacted carbamate and 4-methoxyphenol concentrations were determined by HPLC analysis with a C-18 reverse-phase column using either a gradient mobile phase of 95% dilute H_3PO_4 (1.0 mL of 85% H_3PO_4 in 1.0 L of H_2O)–5% CH_3CN to 5% dilute H_3PO_4–95% CH_3CN over 30 min or isocratic elution with a mobile phase of 87.5% dilute H_3PO_4–12.5% CH_3CN, flow = 1.0 or 3.0 mL/min. The carbamates are stable at the pH of the mobile phase, and therefore injection into the HPLC effectively stops the reaction. The detector was set at 220 nm. The half-life is the time required for 50% conversion of carbamate to 4-methoxyphenol and was calculated by using first-order kinetics. Results in Table I are the average of at least two separate determinations.

Stability of *N*-Methyl-*N*-[2-(methylamino)ethyl]carbamic Acid 4-Methoxyphenyl Ester Hydrochloride (1a) in Murine Plasma. A solution of the carbamate hydrochloride 1a (0.27 mg) in 0.10 mL of dilute H_3PO_4 (1 mL of 85% H_3PO_4 in 1 L of H_2O) was added to a magnetically stirred mixture of fresh murine plasma (1.6 mL) and pH 7.4 phosphate buffer (0.40 mL) preheated to 37 °C to give a carbamate concentration of 5×10^{-4} M. This solution was maintained at 37 °C and pH 7.4 with a Radiometer Copenhagen pH stat. At various intervals, aliquots (25 μL) were removed, quenched in 7% $HClO_4$ (0.20 mL) to stop the reaction, and shaken. After centrifugation (14000 *g*, 8 min) clear supernatant was pipetted from the insoluble pellet and analyzed by the same HPLC method used in the buffer reactions.

Acknowledgment. We are indebted to J. Moreau for elemental analyses, Y. Lee for the pK_a determinations, S. Pitzenberger, J. Murphy, and S. Varga for NMR spectra, and D. Frankenfield for supplying the murine plasma. We also thank W. Randall for helpful advice on determining half-lives of the carbamates and V. Finley for preparation of the manuscript.

Registry No. 1a, 122734-28-5; 1a (base), 122734-36-5; 1b, 122734-30-9; 1b (base), 122734-37-6; 1c, 122754-64-7; 1d, 122734-29-6; 1e, 123183-68-6; 1f, 123183-69-7; 1g, 122734-33-2; 1h, 123183-70-0; 2, 150-76-5; 4a, 112257-19-9; 4c, 122734-34-3; 4d, 122734-32-1; 4d·fumarate, 123183-73-3; 4e, 121492-06-6; 4e·fumarate, 123183-74-4; 4f, 57260-73-8; 4f·fumarate, 123183-75-5; 4g, 75178-96-0; 4g·fumarate, 123183-76-6; 4h, 123183-72-2; 5a, 122734-31-0; 5c, 122734-35-4; 5d, 123183-77-7; 5e, 123183-78-8; 5f, 123183-79-9; 5g, 123183-80-2; 5h, 123183-81-3; 6, 57561-39-4; 7, 123183-82-4; 8, 123183-71-1; di-*tert*-butyl dicarbonate, 24424-99-5; ethylenediamine, 107-15-3; *N*-methylethylenediamine, 109-81-9; 4-methoxyphenyl chloroformate, 7693-41-6; *N,N'*-dimethylimidazolidinone, 80-73-9; *N,N'*-dimethylethylenediamine, 110-70-3; *N*-methylethanolamine, 109-83-1; *N,N'*-diethylethylenediamine, 111-74-0; 1,3-propanediamine, 78-90-0; *N,N'*-dimethyl-1,3-propanediamine, 111-33-1.

Synthesis and Biochemical Studies of 7-Substituted 4,6-Androstadiene-3,17-diones as Aromatase Inhibitors[1]

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Inhibitors of aromatase, the cytochrome P-450 enzyme complex responsible for the biosynthesis of estrogens, may be useful as therapeutic agents for the treatment of estrogen-dependent disease states such as breast and endometrial cancer. Several 7α-thio-substituted androstenediones have proven to be potent inhibitors of aromatase in vitro and in vivo. Recent research efforts have focused on designing aromatase inhibitors with both substitution at C-7 and extended linear conjugation in rings A and B of the steroid nucleus. The targeted compounds, 7-substituted 4,6-androstadiene-3,17-diones 4–10, were prepared by the addition of either Grignard or lithium reagents to 3,3:17,17-bis(ethylenedioxy)-5-androsten-7-one (3). Inhibitory activities of the compounds were evaluated in vitro by enzyme kinetic studies employing the microsomal fraction isolated from human term placenta. 7-Benzyl- and 7-phenethyl-4,6-androstadiene-3,17-dione analogues are effective inhibitors with apparent K_i's of 60.9–174 nM, while the 7-phenyl analogue exhibited an apparent K_i of 1.424 μM. Thus, several 7-substituted 4,6-androstadiene-3,17-diones were prepared and exhibited good competitive inhibition of aromatase in vitro in human placental microsomes.

Aromatase is the cytochrome P-450 enzyme complex responsible for the conversion of androgens to estrogens. Estrogens are involved in reproductive processes and are also implicated in estrogen-dependent disease states such as breast and endometrial cancers. Thus, inhibitors of aromatase may be useful in controlling these physiological processes and disease states. The aromatase inhibitors 4-hydroxyandrostenedione and aminoglutethimide have demonstrated therapeutic effectiveness in the treatment of hormone-dependent breast tumors in both animals[2–4] and humans.[5–7]

Previous work from our laboratory has illustrated that several 7α-thio-substituted derivatives of androstenedione were effective inhibitors of aromatase.[6–12] Among the compounds synthesized, 7α-[(4′-aminophenyl)thio]-4-androstene-3,17-dione (7α-APTA) was found to be one of the most potent inhibitors with an apparent K_i of 18 nM. These aromatase inhibitors have also demonstrated activity in inhibiting aromatase activity in MCF-7 cells[13] and in reducing tumor volumes in the DMBA-induced rat

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Acetaminophen Prodrugs III: Hydrolysis of Carbonate and Carboxylic Acid Esters in Aqueous Buffers

ELISABETH S. RATTIE, ELIE G. SHAMI*, LEWIS W. DITTERT†, and JOSEPH V. SWINTOSKY†

Abstract □ The hydrolysis rates of four carbonate and five carboxylic acid ester prodrugs of acetaminophen were determined in aqueous buffers at various pH's. The hydrolysis reactions of all the compounds except the hemisuccinate were first order in ester and in hydroxyl ion over the relatively alkaline pH ranges studied. The apparent enthalpies of activation were between 18 and 23 kcal./mole. The results suggest that it should be possible to formulate pharmaceutically stable suspensions of this type of acetaminophen prodrugs.

Keyphrases □ Acetaminophen prodrugs—hydrolysis □ Carbonate, carboxylic acid esters of prodrugs—hydrolysis, aqueous buffers □ Hydrolysis rates—acetaminophen prodrug esters □ UV spectrophotometry—analysis

The synthesis, physicochemical properties, and analgesic activities in rats of several prodrug esters of acetaminophen have been previously reported (1–3). These studies showed that significant differences in analgesic potency and duration of action might be expected following the oral administration of carbonate and carboxylic acid esters of various structures. The expected differences were attributable primarily to differences in the rates of dissolution and absorption of the esters following oral administration. In the case of 4-acetamidophenyl 2,2,2-trichloroethyl carbonate, the peak heights and rates of decline of blood levels of acetaminophen in humans depended upon the particle size of the administered powder (4).

Thus, the dose–time–action profiles of drugs that can be converted into carbonate or carboxylic acid esters might be modified at will by: (*a*) selecting appropriate ester structures, and (*b*) controlling the prodrug's particle size in pharmaceutical formulations. The selection of ester structures on the basis of hydrolysis catalyzed by various body enzymes has been discussed (3), but relatively little information on the chemical stability of drugs of this type has been reported. Therefore, the base-catalyzed hydrolysis of several acetaminophen prodrugs was studied to gain information on their relative hydrolytic stability, which would be useful in the formulation of pharmaceutical dosage forms, and to further elucidate their hydrolysis by esterolytic enzymes.

EXPERIMENTAL

Materials—The synthesis and physical properties of the prodrug esters have been described (2). All other chemicals were reagent grade. A Leeds & Northrup model 7401 pH meter and a Cary model 15 spectrophotometer were used.

Buffer Solutions—*Carbonate (pH 10.61, 10.25, 10.0, 9.7, and 9.4)*—These buffers were 0.1 *M* with respect to carbonate ion and were adjusted to ionic strength 0.5 with KCl. The buffers were prepared by dissolving sodium bicarbonate and potassium chloride in distilled water and by adjusting the pH by the dropwise addition of sodium hydroxide.

Phosphate (pH 7.40 and 6.81) and Succinate (pH 5.85 and 5.4)—The method of preparation was similar to that described for the carbonate buffer.

Procedure for Hydrolysis Studies—The hydrolysis rates of the acetaminophen prodrugs in suitable buffers at constant ionic

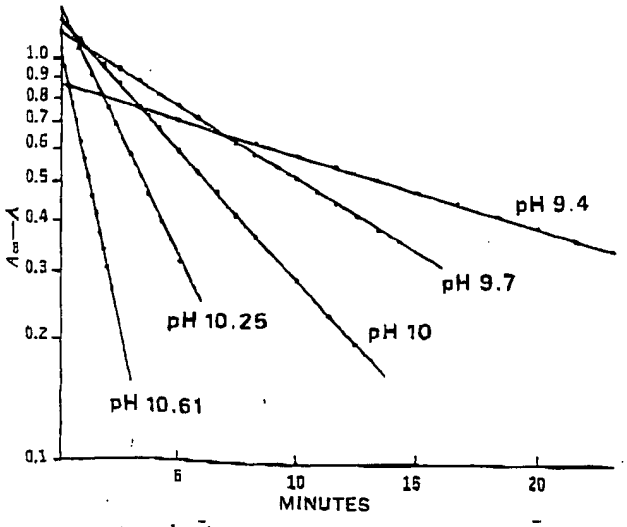


Figure 1—*Plots showing the pseudo-first-order nature of the hydrolysis of 4-acetamidophenyl 2,2,2-trichloroethyl carbonate (II) over the pH range 9.40–10.61 at 25°. The half-lives are shown in Table I.*

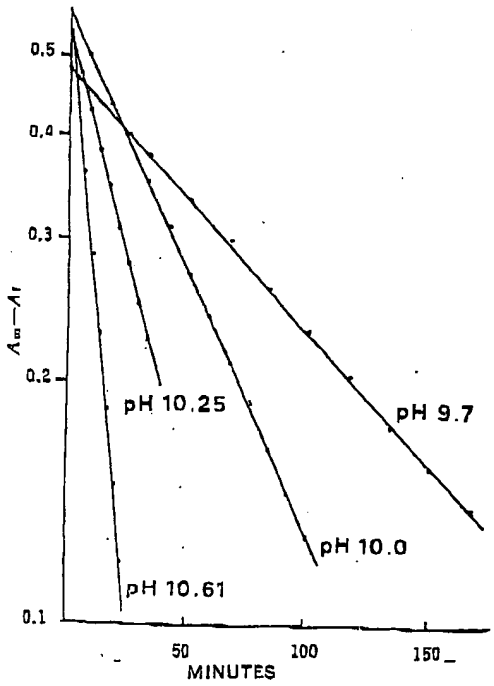


Figure 2—*Plots showing the pseudo-first-order nature of the hydrolysis of 4-acetamidophenyl butyrate (VI) over the pH range 9.70–10.61 at 25°. The half-lives are shown in Table II.*

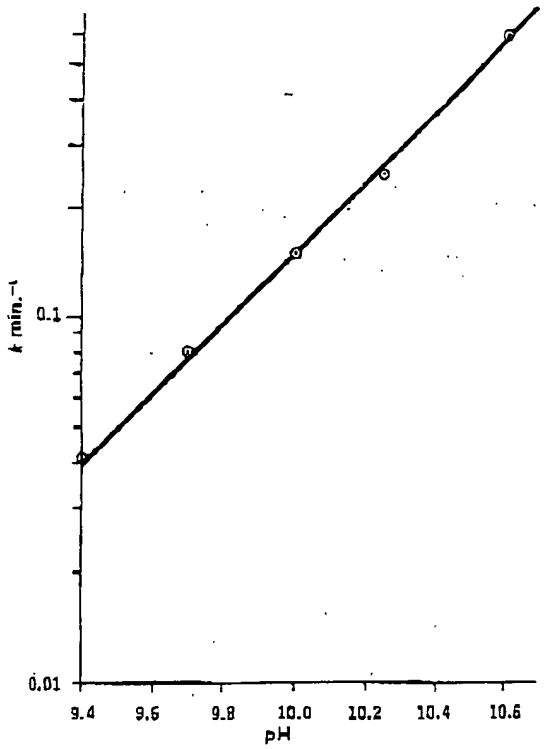


Figure 3—*Plot of pseudo-first-order rate constants* versus *pH for 4-acetamidophenyl 2,2,2-trichloroethyl carbonate (II) over the pH range 9.4–10.61 at 25°.*

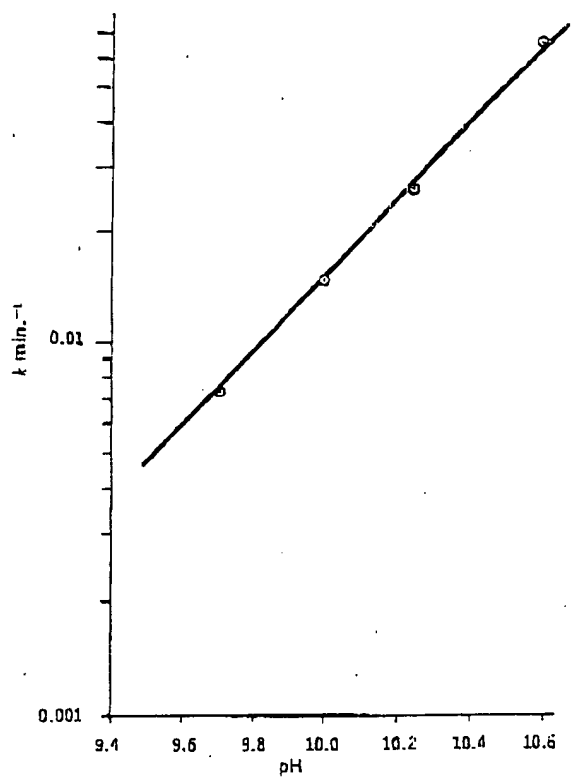
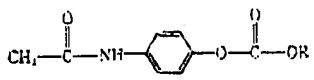


Figure 4—*Plot of pseudo-first-order rate constants* versus *pH for 4-acetamidophenyl butyrate (VI) over the pH range 9.7–10.61 at 25°.*

strength were determined spectrophotometrically by direct UV analysis in the thermostated cell compartment. Fifty milliliters of buffer solution was equilibrated at the approximate temperature in a 50-ml. mixing cylinder. One-half milliliter of anhydrous methanol, containing approximately 0.25 mg. of prodrug, was pipeted into the cylinder, which was then shaken thoroughly. A portion of this mixture was transferred to a 10-cm. sample cell of the spectrophotometer, and the absorbance at 300 mμ (the absorbance maximum of acetaminophen) was followed until no further change in absorbance could be observed (3). All hydrolysis reactions followed pseudo-first-order kinetics, and plots of log $(A_\infty - A_t)$ *versus* time were used to determine the first-order rate constants.

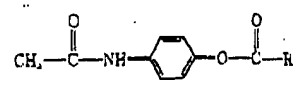
Table I—Hydrolysis Data for Carbonate Ester Prodrugs of Acetaminophen, 25°



R	pH	Buffer System[a]	$t_{1/2}$, min.	$k_{obs.}$, min.$^{-1}$	k_{OH}, l. mole^{-1} min.$^{-1}$[b]
I					
—CH_2—CH_3	10.61	Carbonate	28	0.025	61
	10.25	Carbonate	56	0.012	68
	10.00	Carbonate	103	0.0067	67
	9.70	Carbonate	183	0.0038	76
				Av.	68
II					
—CH_2—C—Cl_3	10.61	Carbonate	1.1	0.62	1.5×10^3
	10.25	Carbonate	2.7	0.25	1.4×10^3
	10.00	Carbonate	4.6	0.15	1.5×10^3
	9.70	Carbonate	8.3	0.082	1.6×10^3
	9.40	Carbonate	17	0.041	1.6×10^3
				Av.	1.5×10^3
III					
—CH_2—$CH(CH_3)_2$	10.59	Carbonate	26	0.027	69
	10.31	Carbonate	48	0.015	74
	9.98	Carbonate	108	0.0064	67
	9.76	Carbonate	168	0.0041	71
				Av.	70
IV					
—$(CH_2)_2$—$N(CH_3)_2 \cdot HCl$	7.40	Phosphate	1.7	0.40	1.6×10^6
	6.81	Phosphate	5.8	0.12	1.9×10^6
	5.85	Succinate	55	0.013	1.8×10^6
				Av.	1.7×10^6

[a] Buffers were 0.1 *M* and adjusted to ionic strength 0.5 with KCl. [b] Calculated fom $k_{obs.}/[OH^-]$, where $[OH^-] = 10^{-}(pk_w - pH)$ and the $pk_w = 13.9965$ at 25°.

Table II—Hydrolysis Data for Carboxylic Acid Ester Prodrugs of Acetaminophen, 25°



R	pH	Buffer System[a]	$t_{1/2}$, min.	$k_{obs.}$, min.$^{-1}$	k_{OH} l.mole^{-1} min.$^{-1}$[b]
V					
$-CH_3$	10.59	Carbonate	6	0.12	320
	10.31	Carbonate	12	0.059	300
	9.98	Carbonate	26	0.026	290
	9.76	Carbonate	42	0.016	320
					Av. 310
VI					
$-(CH_2)_2CH_3$	10.61	Carbonate	11	0.066	170
	10.25	Carbonate	27	0.026	150
	10.00	Carbonate	47	0.015	150
	9.70	Carbonate	93	0.0074	150
					Av. 155
VII					
	10.61	Carbonate	17	0.041	100
	10.31	Carbonate	35	0.020	100
	9.98	Carbonate	78	0.0089	100
	9.76	Carbonate	130	0.0053	110
					Av. 103
VIII					
$-C(CH_3)_3$	10.59	Carbonate	72	0.0097	25
	10.31	Carbonate	148	0.0047	24
	9.98	Carbonate	380	0.0018	20
					Av. 23

[a] Buffers were 0.1 *M* and adjusted to ionic strength 0.5 with KCl. [b] Calculated from $k_{obs.}/[OH^-]$, where $[OH^-] = 10^{-(pk_w - pH)}$ and the pk_w = 13.9965 at 25°.

RESULTS AND DISCUSSION

Log ($A_\infty - A_t$) *versus* time plots for 4-acetamidophenyl 2,2,2-trichloroethyl carbonate (II) and 4-acetamidophenyl butyrate (VI) at various pH's are shown in Figs. 1 and 2, respectively. These figures are typical of the hydrolysis behavior of all the prodrug esters and show that the reactions followed pseudo-first-order kinetics at constant pH. Plots of log *k* (the pseudo-first-order rate constant) *versus* pH were straight lines with slopes essentially equal to 1, showing that the hydrolysis reactions were first order in hydroxyl ion as well as in ester (Figs. 3 and 4). The results of the hydrolysis studies at 25° for the carbonate ester prodrugs are summarized in Table I; those for the carboxylic acid ester prodrugs are summarized in Table II. Essentially the same half-lives as those shown in Tables I and II were obtained in buffers ranging from 0.03 to 0.5 *M* and in solutions of ionic strength ranging from 0.1 to 0.5. Thus, specific buffer catalysis and salt effects are apparently negligible for these hydrolytic reactions.

The results in Table I show that chlorine substitution in the aliphatic alcohol portion of the carbonate ester (II) markedly

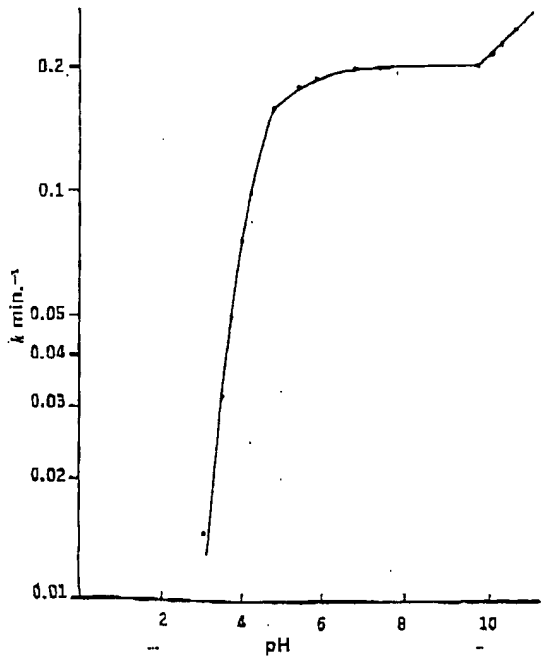


Figure 5—*Plot of pseudo-first-order rate constants* versus *pH for 4-acetamidophenyl hemisuccinate (IX) over the pH range 3.08–10.60 at 25°. The half-lives are shown in Table III.*

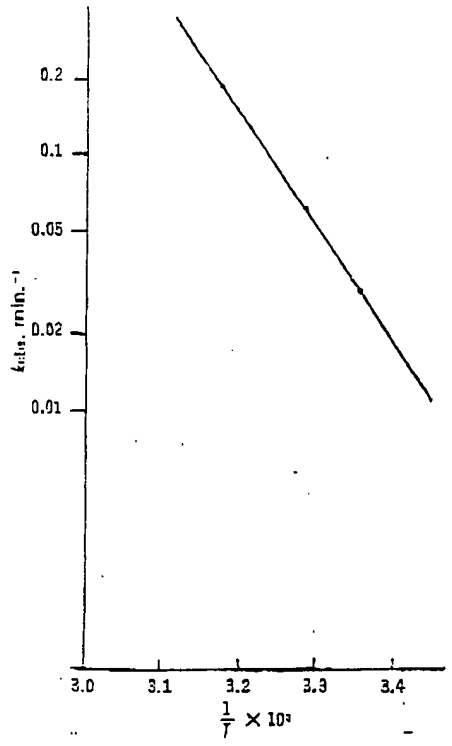
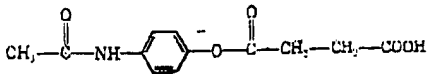


Figure 6—*Arrhenius plot for the hydrolysis of 4-acetamidophenyl ethyl carbonate (I) at pH 10.61 over the temperature range 25–42°. Apparent energy of activation (ΔH) = 21 kcal./mole (Table IV).*

Table III—Hydrolysis Data for Hemisuccinate Ester of Acetaminophen, 25°



(IX)

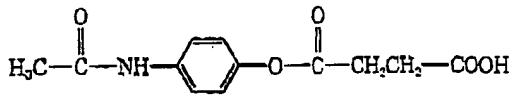
pH	Buffer System[a]	$t_{1/2}$, min.	$k_{obs.}$, min.$^{-1}$
3.08	Citrate	47	0.015
3.5	Citrate	22	0.032
3.7	Citrate	14	0.049
4.0	Acetate	9	0.074
4.25	Acetate	7	0.098
4.75	Acetate	4.5	0.16
5.4	Succinate	3.9	0.18
5.85	Succinate	3.8	0.18
6.81	Phosphate	3.6	0.19
7.41	Phosphate	3.6	0.19
9.72	Carbonate	3.5	0.19
10.05	Carbonate	3.3	0.21
10.27	Carbonate	3.1	0.22
10.60	Carbonate	2.9	0.24

[a] Buffers were 0.1 *M* and adjusted to constant ionic strength 0.5 with KCl.

increased the lability of the ester group to base-catalyzed hydrolysis. The effect was probably due to the electron-withdrawing properties of the chlorine atoms. Chain branching in this alcohol moiety (III) had relatively little influence on the rate of the hydrolysis reaction. Addition of a β-dimethylamino group to the alcohol moiety (IV) caused a dramatic increase in the lability of the ester to base attack. The hydrolysis of IV was first order in hydroxyl ion within the pH region studied (Table I) and can be assigned to an hydroxyl-ion attack on the protonated species.

The results in Table II show that the acetate ester (V) was more labile to base-catalyzed hydrolysis than the ethyl carbonate ester (I). Lengthening the aliphatic chain (VI) or substituting an aromatic hydrocarbon (VII) in the carboxylic acid moiety slightly decreased the lability of the ester group to hydrolysis. Branching of the aliphatic chain near the carboxyl group (VIII) significantly slowed the reaction.

The hydrolysis behavior of the hemisuccinate ester of acetaminophen (IX) was studied extensively because it represents a type of



IX

compound that might display a higher aqueous solubility, especially at higher pH's, than some other prodrug esters. The results shown in Table III and Fig. 5 are very similar to those reported by Gaetjens and Morawetz (5) for phenyl acid succinates. As might be expected, this compound was more stable at low pH's where the free carboxyl group is essentially completely unionized, but it is too labile to be formulated into a suitable pharmaceutical solution under any conditions.

A brief study of the temperature dependency of the hydrolysis reactions showed that, in all cases, the Arrhenius law was obeyed at the pH's studied. A typical Arrhenius plot for Compound I is shown in Fig. 6, and the results for all compounds are summarized in Table IV. The apparent energies of activation are consistent with values previously reported for base-catalyzed ester hydrolyses (5). The conclusion might be drawn from this brief study that stable pharmaceutical suspensions might be formulated from aliphatic carbonate and aliphatic and aromatic carboxylic acid prodrug esters of acetaminophen. A rough extrapolation from pH 10 to pH 5.5, the pH of maximum stability of the acetaminophen amide group

Table IV—Temperature Dependency of the Hydrolysis Reactions of Carbonate and Carboxylic Acid Prodrugs of Acetaminophen

R	pH	$k_{obs.}$, min.$^{-1}$	Temperature	ΔH (kcal./mole)
I	10.61	0.029	25°	21
		0.062	31.5°	
		0.19	42°	
II	9.35	0.044	25°	19
		0.091	31.5°	
		0.022	42°	
III	10.61	0.027	25°	23
		0.055	31°	
		0.024	43°	
IV	5.85	0.013	25°	22
		0.028	31°	
		0.11	43°	
V	10.00	0.026	25°	20
		0.051	31°	
		0.23	45°	
VI	10.61	0.063	25°	19
		0.12	31.5°	
		0.33	42°	
VII	10.25	0.019	25°	18
		0.033	31°	
		0.14	45°	
VIII	10.61	0.0097	25°	20
		0.018	31°	
		0.060	42°	

toward hydrolysis (6), yields t_{90} values of approximately 1, 1, 0.2, 0.4, 0.7, and 3.5 years for Compounds I, III, V, VI, VII, and VIII, respectively. These relatively high t_{90} values for the compounds *in solution*, coupled with their relatively low aqueous solubilities (2) and high doses, suggest that it might be possible to formulate pharmaceutical suspensions with some of the prodrug esters that would retain 90% of their potencies for at least 2 years at room temperature.

The more water-soluble prodrugs, *i.e.*, the *N,N*-dimethylethanolamine carbonate (IV) and the hemisuccinate (IX), are too labile to be formulated in liquid dosage forms under any conditions since they hydrolyze very rapidly at both slightly acidic and slightly basic pH's.

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